Effectors of *Haemophilus ducreyi* Pathogenesis

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ABSTRACT

ROBERT AUBREY FULCHER: Effectors of *Haemophilus ducreyi* Pathogenesis
(Under the direction of Thomas H. Kawula)

Chancroid is a sexually-transmitted infection (STI) prevalent in impoverished regions of the developing world. Caused by the gram-negative bacterium *Haemophilus ducreyi* (*Hd*), an obligate human pathogen, chancroid is characterized by purulent genital skin ulceration and increased risk of acquiring other STI including HIV. Chancroid is treatable with antibiotic therapy but does not elicit protective immunity to subsequent *Hd* infection. To further explore *Hd*-host interactions, we examined two targets: an *Hd* surface protein targeted by the host's modest anti-*Hd* humoral response, and a secreted *Hd* toxin implicated in pathogenesis and immune modulation.

We identified *Hd* outer-membrane protein NcaA as an Oca-family bacterial adhesin. We demonstrated that NcaA mediated *Hd* binding to the host skin protein collagen. Furthermore, we found that NcaA was essential for virulence in human and swine models of chancroid, suggesting an important link between skin colonization and survival in the host.

Most *Hd* isolates secrete cytolethal distending toxin (CDT), a trimeric complex that intoxicates many eukaryotic cell types, including some found in skin (fibroblasts, keratinocytes) and others relevant to immunity (macrophages, lymphocytes). We therefore investigated CDT as a contributor to *Hd* pathogenesis and host immune

modulation. Two independent *Hd* CDT-deletion (ΔCDT) mutants were attenuated in the swine chancroid model, but neither could be complemented, showing that CDT deletion selected for cryptic attenuating mutations. These results support but cannot confirm the importance of CDT to *Hd* pathogenesis. Additionally, pigs inoculated twice with ΔCDT *Hd* were as susceptible to wild type *Hd* challenge as pigs previously inoculated twice with CDT+ *Hd*. Though this finding did not illustrate CDT-mediated immune alteration, ΔCDT strains may not persist sufficiently *in vivo* to elicit immunity regardless of any CDT effect.

Following published findings attributing CDT toxicity to nuclease activity of subunit CdtB, we generated an *Hd* mutant with minimal amino acid variations mapping to the CdtB active site, attempting to circumvent compensatory mutation. This mutant retained CDT toxicity; furthermore, purified mutant CdtB protein retained *in vitro* nuclease activity. Further studies are warranted to address the importance of CdtB nuclease activity and CDT toxicity to *Hd* host-pathogen interaction.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Chapter

LIST OF ABBREVIATIONS AND SYMBOLS

A	alanine
Ap	ampicillin
BHI	brain heart infusion medium
BSA	bovine serum albumin
C.	*Campylobacter*
cat	chloramphenicol acetyltransferase (gene)
CDT	cytolethal distending toxin
CFU	colony-forming unit
Cm	chloramphenicol
D	aspartate (aspartic acid)
DAPI	4', 6-diamidino-2-phenylindole
DIG	digoxigenin
dmB	"double-mutant" *cdtB* / CdtB
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTH	delayed-type hypersensitivity
E.	*Escherichia*
ECM	extracellular matrix
EDD	estimated delivered dose
ELISA	enzyme-linked immunosorbent assay

EtBr	ethidium bromide
g	gram
gDNA	genomic DNA
GUD	genital ulcer disease
H.	*Haemophilus*
H	histidine
HEPES	2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid
HIV	human immunodeficiency virus
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Kn	kanamycin
L	liter
lacZ	gene encoding β-galactosidase
LacZ	β-galactosidase
mAb	monoclonal antibody
mg	milligram
mL	milliliter
mM	millimolar
$Ni^{2\pm}$-NTA	nickel-charged nitriloacetic acid
Oca	oligomeric coiled adhesin

OMP	outer membrane protein
p. i.	post-inoculation
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + 0.05% Tween-20
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
Q	glutamine
RNA	ribonucleic acid
RT	reverse transcriptase *or* room temperature
S	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STI	sexually-transmitted infection
TAE	tris-acetate-EDTA buffer
tmB	"triple-mutant" *cdtB* / CdtB
Vc	vancomycin
VCN	vancomycin-colistin-nystatin medium supplement
wtB	wild type *cdtB* / CdtB
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	anti-
Δ	deletion
μg	microgram

μM	micromolar
μL	microliter
::	gene insertion

CHAPTER 1

INTRODUCTION

Chancroid is a sexually transmitted disease that is largely unrecognized by the American public, and rarely seen by health professionals outside developing countries. It is caused by the gram-negative bacterium *Haemophilus ducreyi*, a fastidious obligate human pathogen. Over the last few years, only a handful of chancroid cases have been reported in the United States. Despite its relative scarcity in developed nations, chancroid is prevalent in many relatively impoverished areas of the world. These regions also suffer from poor public health care and limited access to antibiotic therapies, which are still very effective against chancroid. Chancroid patients are not afforded protective immunity following infection. Those repeatedly exposed to *H. ducreyi* can contract the disease multiple times. Because the transmission rate is high, most chancroid patients in endemic areas have a high probability of being infected more than once. The most troubling health burden of chancroid comes not from the pathology that it causes, but from the increased susceptibility of its host to infection with other sexually transmitted infections (STI), many of which are neither as benign nor as treatable. For these reasons, it is important to understand the means by which chancroid causes disease, modulates immunity, and facilitates acquisition of other pathogens.

History of chancroid

Chancroid is a member of the family of genital ulcer diseases (GUD) including syphilis, herpes and donovanosis, among others. Chancroid was first recognized as a distinct GUD in 1852, when French dermatologist Leon Bassereau distinguished the chancroid ulcer from the similar chancre seen in syphilis (11). Although Bassereau did not identify the causative agent or agent(s), making a distinction between syphilis and chancroid lesions was a testament to his skill; even today, it is thought that many cases of chancroid are misdiagnosed and improperly reported as other GUD such as syphilis or herpes (78). Over three decades later, French research assistant Auguste Ducrey began a detailed study of the *ulcus molle*, or "soft chancre" associated with chancroid. Though he was unable to culture the agent on laboratory media, he showed that chancroid lesions could be serially passaged along the forearm skin of patients by autoinoculation with exudate from the primary ulcer (25). After several passages, Ducrey isolated the specific agent: a "streptobacillary rod with a clumping or chaining morphology" (26).

Some speculation remains as to which scientist or scientists first isolated "Ducrey's bacillus", and this controversy has been reviewed in detail (4, 33). Bezancon, however, published verification of Koch's postulates by isolating the bacillus from a chancroid lesion, obtaining a pure culture on blood agar, inoculating healthy subjects with this culture, and re-isolating the causative agent from the experimental lesion (15). Ducrey's bacillus was later classified in the genus *Haemophilus* based on its gram-negative phenotype, its coccobacillary morphology and its metabolic requirement for factors X

(hemin) and V (nicotinamide adenine dinucleotide) (4). Various studies involving DNA homology (18) and 16S ribosomal RNA sequencing (21) have since placed *H. ducreyi* within the family *Pasteurellaceae*, distinct from the main grouping of true *Haemophilus* species. The closest relatives of *H. ducreyi* reside within the *Actinobacillus* and *Pasteurella* genera (23).

Chancroid pathology in the human host

Haemophilus ducreyi is an obligate human pathogen, occupying no other known environmental or animal vector niche (57). It is transmitted and acquired solely by direct physical contact with *H. ducreyi* bacteria shed from an infected individual via the chancroid lesion. *H. ducreyi* enters a new host through microscopic tears in the epidermis, such as those sustained during sexual intercourse. As few as 10 to 100 bacteria are thought to be required to initiate infection, as determined in an experimental human chancroid model (81, 83). Within 4 to 10 days a raised red papule forms at the site of entry, and over the next few days develops into a pustule. Most people are unaware of infection through these first stages, but eventually the pustule ruptures to form an acutely painful chancroid ulcer, prompting most chancroid patients to seek medical intervention.

Lesion pathology associated with *H. ducreyi* infection has been well documented (4, 33, 57, 58, 81), and is summarized here. The typical chancroid ulcer is irregular in shape, bordered by a reddened edge but with limited erythema of surrounding skin (Figure 1.1A). The floor of the ulcer has a rough granular appearance, and bleeds easily

when disturbed or abraded. Chancroid ulcers exude copious purulent material and are highly vascularized, bleeding easily upon abrasion. Men most often develop lesions on the foreskin, glans, or distal shaft of the penis, whereas lesions in women develop on the external genitalia. Ulcers can also occur on the vaginal wall or cervix, but these lesions are typically not painful and thus often remain untreated. Like the subjects of Ducrey's early research, naturally infected patients may generate secondary lesions on their hands or forearms by autoinoculation of bacteria from the primary lesion. This peculiarity of chancroid demonstrates that protective immunity is not elicited by *H. ducreyi* infection.

If left untreated, a given chancroid lesion will resolve within one to two months following ulcer formation. *H. ducreyi* does not persist within the body after resolution, nor does it cause systemic infection, though in some instances infection may ascend to the draining inguinal lymph node, leading to an intense inflammatory response that produces an inguinal bubo. This complication occurs more often in men than in women, and without drainage by aspiration or incision, buboes can rupture (Figure 1.1B). Interestingly, aspirate from chancroid-associated buboes contains relatively few *H. ducreyi* bacteria as compared to purulent matter isolated from the primary lesion. In extraordinary cases, prolonged untreated cases of chancroid in men can produce anatomical damage requiring circumcision or surgical reconstruction.

Chancroid pathology is largely mediated by the host's immune response to *H. ducreyi.* Histological examination of the chancroid ulcer shows a profound cellular infiltrate chiefly populated by polymorphonuclear leukocytes (PMN). The primary role

of PMN in chancroid is to generate reactive oxygen and nitrogen species in an attempt to keep *H. ducreyi* in check; this sustained influx of PMN to the lesion site produces the characteristic papule-pustule-ulcer progression. *H. ducreyi* is especially effective at inducing of IL-8 expression by skin cells (96); IL-8 is a chemoattractant for both PMN and T lymphocytes (61) which are also prevalent within the chancroid lesion. *H. ducreyi* appears to withstand the oxidative burst and, in fact, replicates within the base of the lesion. The presence of T lymphocytes (predominantly $CD8^+$) and macrophages within the lesion along with elevated TNFα, IL-8, and IFNγ (46) have led researchers to describe the anti-*H. ducreyi* response as a delayed-type hypersensitivity (DTH) reaction (64). DTH reactions are recall responses requiring an initial priming exposure to the target antigen(s); it is not known whether cross-reactivity to other related bacterial antigens potentiates a bonafide DTH response against *H. ducreyi*.

Despite the ferocity of the innate immune response to *H. ducreyi* infection, the adaptive immune response to *H. ducreyi* appears much less effective. Because *H. ducreyi* is an extracellular pathogen (12-14), durable immunity would likely involve a humoral response. People who have ulcerated lesions for less than one week may not seroconvert, but people with ulcers of 8-day duration or higher mount an *H. ducreyi*-specific antibody (IgM, IgG and IgA) response that increases during infection and persists (predominantly IgG) for at least 18 months afterward (6, 69, 72). However, even people with measurable anti-*H. ducreyi* antibody titers are not immune, as evidenced by susceptibility to repeated autoinoculation used in early chancroid studies identifying chancroid (25) as well as in

early chancroid diagnosis (37). It is unclear whether the low efficacy of humoral immunity results from low immunogenicity of the bacterium, preferential generation of antibodies against ineffective *H. ducreyi* targets, or the disproportionately large contribution of the innate immune response to the overall chancroid syndrome. It is possible that *H. ducreyi* has developed mechanisms to actively modulate host immunity, amplifying the cellular response at the expense of the humoral response.

In the United States, herpes is the most common GUD, followed by syphilis, then chancroid and other bacterial GUD. Direct diagnosis of chancroid is problematic; no FDA-approved nucleic acid test currently exists for *H. ducreyi*, and culture is difficult because the bacterium does not survive well in routine transport media and must be directly plated onto enriched media (50, 94). Detection frequency from ulcer swab cultures is approximately 40% (50). Though some labs have developed certified in-house PCR-based *H. ducreyi* diagnostics, chancroid is most often diagnosed by excluding syphilis and herpes. A patient presenting ulcerative genital lesions should be tested for syphilis by dark-field microscopy (to detect *T. pallidum* spirochetes in lesion exudate) and for herpes simplex virus (HSV) by culture or serology. Negative results for these tests yield a presumptive diagnosis of chancroid. Accompanying inguinal lymphadenopathy is also highly suggestive of *H. ducreyi* infection. Further complicating diagnosis is the fact that many GUD patients are simultaneously infected with more than one of these pathogens (94).

Chancroid and public health

Chancroid is a rare disease in industrialized nations, far eclipsed by herpes as the most common GUD (58). Surveillance data from 2004 indicate that only 17 chancroid cases occurred in the United States (19), though difficulty in culturing *H. ducreyi* and its potential misdiagnosis as syphilis likely contribute to a lower apparent frequency (78). Limited outbreaks within the United States have occurred within the past two decades, and have been associated with communities in which drug use and HIV are common (24, 55).

Chancroid is most prevalent in the developing world, primarily impoverished areas of Southeast Asia, Sub-Saharan Africa, South America and the Caribbean (58, 59, 71). In these countries, endemic chancroid accounts for the majority of GUD cases (58). HIV is also common in these areas, and shares an "epidemiological synergy" with GUD (90). HIV-positive individuals with GUD more efficiently transmit HIV to their sexual partners, and GUD in HIV-negative individuals can increase their risk of HIV seroconversion by up to 18-fold upon sexual contact with an infected partner (74). The presence of $CD4^+$ T lymphocytes within chancroid ulcers provides a potential ready target for HIV acquisition (83) and supports other findings suggesting that the cooperative interaction between chancroid and HIV may be greater than that in other GUD (52). HIV also increases the potential for *H. ducreyi* transmission, producing more persistent chancroid lesions (45, 68). With a transmission rate approaching 70 percent (66), the

important role of chancroid in the global dissemination of HIV clearly warrants efforts to control or eradicate *H. ducreyi*.

Antibiotic therapy remains effective against *H. ducreyi* infection. Current recommended therapy consists of single-dose azithromycin (1 g) or ceftriaxone (250 mg IM), or a three- to seven-day course of ciprofloxacin or erythromycin, although some isolates resistant to the latter two drugs are emerging. Single-dose therapies are particularly beneficial in ensuring patient compliance, however these drugs are relatively expensive and are not often dispensed in areas where chancroid is most common (94). In addition, chancroid in HIV-patients is often poorly responsive to antibiotic treatment (45, 68).

The primary obstacles to chancroid eradication are poor access to health care and the synergistic relationship between chancroid and HIV. Antibiotic treatment failure is much more common in HIV-positive chancroid patients, whose lesions may be more severe and persistent than those in immunocompetent individuals (52, 68). Though some laboratory animal model studies have generated hopeful *H. ducreyi* vaccine candidates, no vaccine is currently available. As an obligate human pathogen, it is conceivable that with aggressive implementation of such a vaccine, as well as improved health care and education, chancroid eradication may be feasible.

Chancroid model systems

Model systems have been developed to better understand the means by which *H.*

ducreyi causes disease. Cell lines derived from skin keratinocytes (HaCaT) and fibroblasts (Hs27), as well as the more common HeLa and COS-7 lines have been employed to study virulence of *H. ducreyi* as well as purified components thereof. More complex stratified co-culture systems have also been used to study *H. ducreyi* in an environment that more closely mimics the stratified structure of skin (39, 96). Although focused and convenient, *in vitro* studies lack the ability to assess pathogenesis in the context of an intact host response. To better characterize *H. ducreyi* pathogenesis, several animal models of chancroid have been developed. In both *in vitro* and *in vivo* model systems, work with various isolates and genetically-modified strains of *H. ducreyi* has revealed much about the molecular pathogenesis of chancroid.

The rabbit model is probably the most long-standing animal model for chancroid. Ducrey attempted unsuccessfully to culture *H. ducreyi* by intradermal injection into rabbits (25, 26) but a temperature-dependent rabbit model later refined by Purcell *et al.* (67) produced a more consistent and representative experimental chancroid lesion. In this model, rabbits are housed at 15-17°C to provide a more suitable *in vivo* growth temperature for *H. ducreyi*, which grows optimally at approximately 33°C. The number of *H. ducreyi* bacteria required to elicit a lesion in the rabbit model is relatively high, and after naïve rabbits are inoculated with *H. ducreyi* (34) or with purified *H. ducreyi* lipooligosaccharide or pilin (22), they mount a vigorous antibody response that protects from subsequent infection. While relatively inexpensive and useful for identifying virulence factors that have a profound effect on initiation of *H. ducreyi* infection (82, 88,

91), the facile development of immunity to *H. ducreyi* in the rabbit model contrasts sharply with natural chancroid in humans, making this model less suitable for characterizing the immune response directed at *H. ducreyi*.

A primate model of chancroid was developed by Totten *et al.* (87) to approximate more closely the human host environment to study *H. ducreyi* pathogenesis. This model provided the unique opportunity to inoculate genital skin of both male and female macaques to more accurately study *H. ducreyi* infection within the context of the anatomical site of naturally-acquired chancroid. Lesion pathology and immune response in macaques appeared to be congruent with that in humans, including the formation of ulcerated lesions harboring viable *H. ducreyi* over the span of three weeks, and the induction of inguinal lymphadenopathy in a subset of the male macaques. However, females were not susceptible to *H. ducreyi* infection (87). The primary disadvantage of this model is the extremely high cost of using primates as research animal subjects.

Spinola *et al.* developed a human challenge model of chancroid, citing the obvious advantage of studying *H. ducreyi* infection within its natural host. Healthy volunteers are inoculated on the upper arm with a standard allergy test applicator, and disease progression is monitored using a variety of measurements including lesion formation frequency, histopathology, culture of viable organisms from lesions, and comparison between mutant and parental *H. ducreyi* strains (83). The course of lesion development closely mimics the natural disease and volunteers do not become immune to infection, even after second inoculation (9). Pustule formation is a mandatory endpoint for

experimental infection due to the discomfort involved, so lesions at the late pustular or ulcerative stages cannot be modeled in humans. This restriction may preclude development of an immune response similar to that seen in natural infection. Furthermore, recombinant *H. ducreyi* strains containing mobile antibiotic resistance elements may not be tested in the human model. Despite these restrictions, the human model is an invaluable source of information regarding the contributions of many *H. ducreyi* gene products to chancroid pathogenesis (7, 8, 13, 32, 65, 85, 86).

The swine model of chancroid capitalizes on the anatomical and physiological similarities between pigs and humans. First described by Hobbs *et al.* (40) this experimental approach employs young pigs inoculated with *H. ducreyi* in a manner similar to that used in the human model. This model uses the dorsal aspect of the pig ear, where skin thickness closely approximates that of human thin skin, and the skin temperature is near the ideal of 34-35°C. Lesion formation is somewhat accelerated relative to humans, but the histopathology of the resulting ulcers is very similar to natural chancroid. Each inoculation site can be analyzed as an independent lesion, as was shown in the human model (10), and the surface area of the pig ear allows simultaneous inoculation of multiple sites. Thus, the pig model produces a large amount of data from each animal. Furthermore, like humans, pigs do not become immune following single infection, remaining susceptible for multiple rounds of inoculation. Studies of immune response in this model are somewhat hampered by the fact that swine immunology is less well-defined than that of the canonical mouse or human. Furthermore, the pig model

system is somewhat sensitive to high temperature, and pigs must be housed at an ambient temperature no greater than 68°F. Overall, we have found the swine model to be a workable compromise between the host species parity of the human model and the ability to allow lesions to progress to resolution, if warranted.

The arsenal of *Haemophilus ducreyi*

In order to survive within the human host, *H. ducreyi* employs a range of molecular tools. Most of these virulence factors address primary concerns of a bacterial pathogen: acquisition of nutrients, adherence to host tissues, and evasion of host defenses. Over the past several decades, with the help of many collaborative efforts, much knowledge has been gained regarding these virulence determinants in *H. ducreyi*. To date, eight gene products or loci have been identified (8, 20, 31, 41, 42, 48, 82, 88) that are indispensable for *H. ducreyi* infection in one or more experimental chancroid models, including one which will be discussed later in Chapter 3. Some examples of these virulence factors are summarized below.

Nutrient acquisition

As an obligate human pathogen, *H. ducreyi* is keenly adapted to its natural habitat within host skin tissue. The detriment of this adaptation is that *H. ducreyi* requires a very rigid set of parameters for growth. Iron acquisition is of particular importance; a primate model study showed that providing macaques with supplemental iron reduced the

number of *H. ducreyi* bacteria required to form chancroid lesions (84). As a predominantly extracellular pathogen, *H. ducreyi* must live within a host environment that is practically devoid of non-sequestered iron. *H. ducreyi* has evolved the ability to acquire iron from a variety of heme-containing sources within the host, such as hemin, hemoglobin and catalase (49). Elkins *et al.* characterized the hemoglobin-binding protein HgbA (27, 28), which mediates TonB-dependent (30) iron scavenging from heme sources in the host. Not only is HgbA expression required for *H. ducreyi* growth in humans (8), but immunization of pigs with purified HgbA produced a humoral response that is highly bactericidal *in vitro* and completely protected immunized pigs against *H. ducreyi* challenge (2). This result demonstrates the importance of iron acquisition to *H. ducreyi* survival *in vivo*, and shows that HgbA is a very promising immunogen for incorporation into a chancroid vaccine.

Adherence to host tissues

Some *Actinobacillus* species carry genes encoding fimbria-like proteins that enable adherence to a variety of inert and biological surfaces (43, 44). The *flp* operon is a homologous multigene cluster found in *H. ducreyi* that is required for the formation of microcolonies *in vitro* (60). Because adherence to host tissue is often an important preliminary step in establishing bacterial infection, strains of *H. ducreyi* unable to manufacture Flp proteins were assayed for virulence. Excision of *flp1* and *flp2* (Flp structural subunit genes) had no impact on *H. ducreyi* virulence in the rabbit chancroid

model (60), but inactivation of *tadA*, a central *flp* locus gene and putative energizer of the *flp* system, resulted in an attenuation in the human challenge model (82). To date, the *flp1 flp2* mutant has not been tested in humans, and it is not known whether the loss of TadA or the polar effect of the *tadA* mutation on downstream genes is responsible for the attenuated phenotype of this mutant (60).

As part of the innate immune system, complement molecules present in normal non-immune serum can attach to certain conserved bacterial structures, activating the antibacterial complement cascade in the absence of pathogen-specific antibodies, known as the "alternative pathway" of complement fixation (70). *H. ducreyi* proteins DsrA and DltA are involved in *H. ducreyi* serum resistance, preventing initiation of the alternative pathway against *H. ducreyi* and thereby deflecting one arm of innate host immunity (29, 48). Survival of *H. ducreyi* strains unable to express DsrA was strongly inhibited in pigs and humans (17, 20), and *dltA* mutants had diminished but not absent viability in experimentally infected humans (41). Furthermore, DsrA facilitates binding to the extracellular matrix (ECM) protein vitronectin as well as human keratinocytes *in vitro*, though these two functions do not appear to be directly linked (20). DltA, a carbohydrate-binding lectin, was identified by its binding affinity toward lactose-containing sugar moieties (48). Though a direct link between DltA carbohydrate binding and serum resistance has not been established, DltA lectin activity may have a positive impact on bacterial binding to host glycoprotein structures (48).

DsrA is a member of the oligomeric coiled adhesin (Oca) family of bacterial outer membrane proteins. Also referred to as type Vc oligomeric autotransporters (35), Oca protein monomers assemble to form a stalk-like coiled stem structure, terminating in a globular "head" domain. Like *H. ducreyi* DsrA, Oca family members found in pathogenic *Yersinia*, *Moraxella*, and *Escherichia* species have been shown to confer both ECM adherence and serum resistance phenotypes (1, 54, 73, 77). We identified another Oca-family adhesin in *H. ducreyi* that is homologous to DsrA, and its contribution to virulence is discussed in greater detail in Chapter 3 of this work.

Evasion of host immunity

The abundance of inflammatory cells within the chancroid lesion creates a very hostile environment for bacteria. They are subject to engulfment by professional phagocytic cells or attacked by reactive oxygen and nitrogen intermediates produced by PMN. *H. ducreyi* expresses proteins that subvert both these defense mechanisms to enable survival *in vivo*. Researchers noted in several instances that *H. ducreyi* appears to evade engulfment by professional phagocytic cells (12, 13), and later showed that this antiphagocytic activity extends to nearby inert foreign particles as well (3, 92). First identified as large supernatant proteins found in *H. ducreyi* broth cultures (89), LspA1 and 2 were found to be essential in both rabbit (88) and human (42) models of chancroid. Mock *et al*. illustrated that the LspA proteins interfere with Src tyrosine kinase signaling

in macrophages, thereby blocking phagocytosis (56), which hampers host efforts to clear the chancroid lesion of *H. ducreyi*.

Defense against the oxidative burst is provided by enzymes that break down toxic reactive species into less toxic products. Superoxide dismutase, encoded by the *sodC* gene, is responsible for the detoxification of the superoxide radical (75). Located in the periplasmic space, SodC uses a coordinated Cu-Zn catalytic center to convert superoxide O_2^- into the less reactive species hydrogen peroxide (H_2O_2) and H_2O. In this manner, *H. ducreyi* circumvents intracellular damage caused by this PMN-generated antibacterial response and is able to withstand oxidative stress within the chancroid lesion. San Mateo *et al.* elegantly showed that an *H. ducreyi* strain unable to express SodC was profoundly attenuated in the pig model of chancroid; cyclophosphamide-induced depletion of PMN in the host prior to inoculation rescued the virulence phenotype of the *sodC* mutant (76). Discordantly, SodC was found nonessential to *H. ducreyi* virulence in the human chancroid model (16). The cause of this difference is unknown, but it may reflect differences in the interaction of swine versus human immune cells with *H. ducreyi*, or an artificially elevated contribution of SodC to *H. ducreyi* virulence in a non-natural host (16).

Several *H. ducreyi* gene products that seem to have direct correlation to virulence have proven to be nonessential for virulence in animal models of chancroid (7, 95). Two of these proteins are the contact hemolysin HhdB and the cytolethal distending toxin (CDT). The contact hemolysin is a *Serratia*-type pore-forming toxin (36) expressed from

the *hhdBA* locus, with HhdA facilitating the transport of the active subunit HhdB across the cytoplasmic membrane (63). HhdB is active against human foreskin fibroblasts (5), the HaCaT keratinocyte cell line (62), macrophages, and lymphocytes (93). This activity is not diffusible; HhdB is associated with the bacterial surface, and requires contact between *H. ducreyi* and the target cells to induce cytotoxicity (5). In contrast, CDT is a soluble factor actively secreted by *H. ducreyi.* CDT is a heterotrimeric complex of CdtA, CdtB, and CdtC polypeptides capable of intoxicating a wide range of eukaryotic cell types (reviewed in (80)). CDT causes cell-cycle arrest in target cells, and its name is derived from the cells' distended morphology as growth continues without division. Apoptotic cell death follows intoxication, usually after 24 to 36 hours. CDT homologs expressed by other gram-negative bacteria have been implicated as potential modulators of host immunity (38, 79), making CDT an especially intriguing candidate for study with regard to *H. ducreyi* pathogenesis. An *H. ducreyi cdtC hhdB* double mutant was tested in both swine (Kawula Lab, unpublished data) and human (95) chancroid models, and its virulence was found to be equivalent to wild type. Thus, the current literature reports both hemolysin and CDT as being dispensable for *H. ducreyi* virulence. Some controversy exists around this assessment of the role of CDT in *H. ducreyi* pathogenesis. Though the *cdtC hhdB* double mutant used in the human challenge model cannot form intact CDT holotoxin (a CdtA-CdtB-CdtC trimer) it does express CdtB, the primary toxigenic subunit. Delivery of CdtB alone into cultured cells can recapitulate cytopathology seen in CDT holotoxin-exposed cells (47, 51, 53). Therefore, we asserted

that a *cdtC*-deficient strain of *H. ducreyi* may have residual toxicity, and was inappropriate for use as a "CDT-null" mutant for evaluation of CDT toxicity in models of *H. ducreyi* pathogenesis. Continued investigations to this effect are discussed at greater length in Chapter 4.

In the context of global public health, *H. ducreyi* and its contribution to the spread of epidemic sexually-transmitted infections like HIV are a serious cause for concern. This pathogen is keenly adapted to survive exclusively within the human population, and does so by controlling or avoiding host defenses. By studying the means by which *H. ducreyi* achieves its molecular subterfuge, we can begin to design intelligent ways to counteract *H. ducreyi* virulence, build immunity to chancroid, and ultimately eradicate this serious threat to people of the developing world. The following chapters describe our efforts to elucidate *H. ducreyi* pathogenesis using the swine experiment model of chancroid to explore 1) the nature of the humoral response against *H. ducreyi*, 2) a novel determinant of *H. ducreyi* adherence to host collagen, and 3) the heretofore underestimated contribution of CDT to *H. ducreyi* infection.

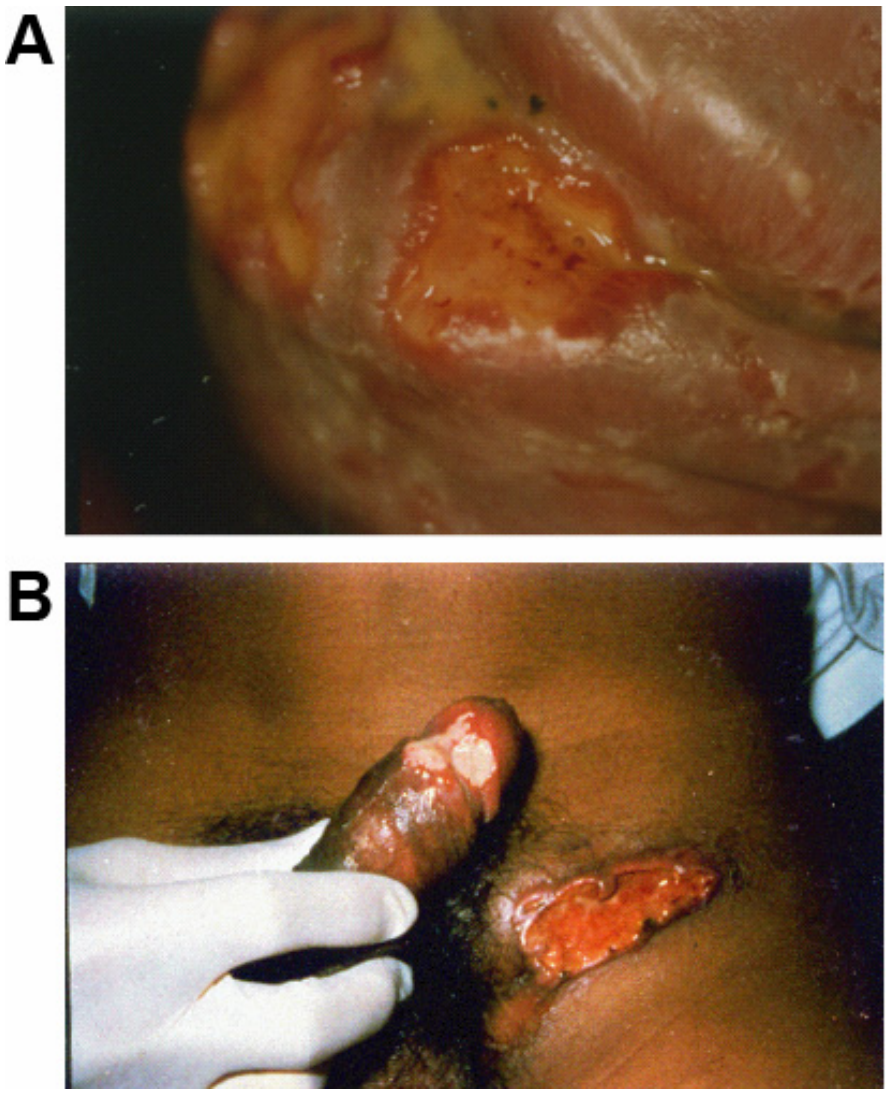

Figure 1.1. Natural chancroid lesions in the human host.
Panel A illustrates a cluster of chancroid ulcers near the penile frenulum. Note the irregular shape, purulent appearance, and bleeding points within the floor of the lesion. Panel B shows both the primary frenular chancroid lesions as well as rupture of an inguinal bubo secondary to ascension of the inflammatory response to the draining lymph node.

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CHAPTER 2

A Humoral Immune Response Confers Protection against *Haemophilus ducreyi* Infection

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Haemophilus ducreyi is the etiologic agent of the sexually transmitted genital ulcer disease chancroid. Neither naturally-occurring chancroid nor experimental infection with *H. ducreyi* results in protective immunity. Likewise, a single inoculation of *H. ducreyi* does not protect pigs against subsequent infection. Accordingly, we used the swine model of chancroid infection to examine the impact of multiple inoculations on a host's immune response. After three successive inoculations with *H. ducreyi*, pigs developed a modestly protective immune response evidenced by the decreased recovery of viable bacteria from lesions. All lesions biopsied 2 days after the first and second inoculations contained viable *H. ducreyi* cells, yet only 55% of the lesions biopsied 2 days after the third inoculation did. Nearly 90% of the lesions biopsied 7 days after the first inoculation contained viable *H. ducreyi* cells, but this percentage dropped to only 16% after the third inoculation. Between the first and third inoculations, the average recovery

of CFU from lesions decreased approximately 100-fold. The reduced recovery of bacteria corresponded directly with a fivefold increase in *H. ducreyi*-specific antibody titers and the emergence of bactericidal activity. These immune sera were protective when administered to naïve pigs prior to challenge with *H. ducreyi*. These data suggest that pigs mount an effective humoral immune response to *H. ducreyi* after multiple exposures to the organism.

Introduction

Chancroid is a sexually transmitted infection often seen in resource-poor tropical areas (34) and is a prevalent genital ulcer disease in certain areas of Asia, Africa, and Latin America (31, 34, 35). As both cross-sectional cohort studies and prospective longitudinal studies indicate that all genital ulcer disease (13, 23, 41, 51) and chancroid in particular (31, 33) increase the risk for sexual transmission and acquisition of human immunodeficiency virus type 1 (HIV-1), control of chancroid could provide an effective intervention strategy against the spread of human immunodeficiency virus type 1 (18, 21, 27, 34, 37, 41, 44, 52). Control of chancroid is complicated by the fact that natural chancroid infection does not appear to protect against subsequent infection (6, 25, 43). Concordantly, a single experimental infection with *Haemophilus ducreyi* does not protect human volunteers against subsequent experimental challenge (4, 49).

Humans mount what appears to be a delayed-type (type IV) hypersensitivity reaction in response to *H. ducreyi* (22, 32, 38, 49). This response is neither protective against future infection nor effective at clearing chancroid infections as lesions can persist for weeks or months and ulcer resolution is often incomplete in the absence of antibiotic therapy (34). One possible reason for the ineffective nature of this response is that cell-mediated immunity is highly effective at killing intracellular bacteria and viruses (30), yet the majority of *H. ducreyi* present in chancroid lesions are extracellular (5).

While the delayed-type (type IV) hypersensitivity response appears ineffective at preventing future chancroid infections, it is unclear what sort of response would be protective. The extracellular existence of the bacteria suggests that a humoral immune response could be protective against infection. We repeatedly exposed pigs to *H. ducreyi* in an attempt to elicit and identify a protective immune response in the swine model of chancroid. Pigs, like humans, are not protected from subsequent infection by a single exposure to *H. ducreyi*. However, after three inoculations at 14-day intervals, pigs developed a modest but significant level of protective immunity against *H. ducreyi*. Protection was defined not as an absolute block of infection but rather as a reduction in disease severity as indicated by reduced recovery of viable *H. ducreyi* cells. Passive transfer of immune serum protected naïve animals against challenge with *H. ducreyi*. These results from the swine model support the idea that humoral immunity to *H. ducreyi* could provide protection against infection by this organism.

Materials and Methods

Bacterial inoculum preparation.

The inoculum was prepared and quantified as previously described (29, 45). Briefly, *H. ducreyi* strain 35000HP (gift from Stanley Spinola) was grown from a freezer stock and passed once on chocolate agar plates containing 1.5% (wt/vol) Bacto agar, 2.5% brain heart infusion (BHI), 1% hemoglobin, 1% IsoVitaleX (all from Becton Dickinson, Cockeysville, Md.), 5% newborn calf serum, and 5% fetal bovine serum (Life Technologies, Inc., Rockville, Md.) at 35°C in a humidified incubator with 5% CO_2. Vancomycin, when used, was added to growth media at a concentration of 3 μg/ml.

Animals.

Juvenile female crossbred (Yorkshire-Landrace crossed with Hampshire-Duroc [hereafter, Yorkshire cross]) pigs were employed as previously described (29). Pigs were housed in individual enclosures at North Carolina State University College of Veterinary Medicine in a P2 containment facility accredited by the American Association for Accreditation of Laboratory Animal Care. All pigs were 6 weeks old at the beginning of the study. Animals were sedated for procedures with 0.3 ml of a TKX cocktail per 22.7 kg of body weight. The cocktail consisted of tiletamine HCl-zolazepam HCl (each, 50 mg/ml), (Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa), ketamine HCl (50 mg/ml) (Fort Dodge Laboratories), and xylazine (50 mg/ml) (Miles Laboratories, Shawnee

Mission, Kans.). Atropine sulfate (Phoenix Scientific Inc., Joseph, Mo.) was given in the amount of 0.5 ml to 1 ml, depending on the size of the animal, in order to slow bronchial secretion and prevent aspiration.

Inoculation.

Eight sites on the dorsal side of each ear for a total of 16 sites per pig were inoculated with Multi-Test multiple skin test applicators (Lincoln Diagnostics, Decatur, Ill.) as previously described (29, 49). Two sites per ear were inoculated with the Multi-Test applicators loaded with each of the following: 10^6 CFU of *H. ducreyi* 35000HP, 10^7 CFU of *H. ducreyi* 35000HP, 10^6 CFU of heat-killed *H. ducreyi* 35000HP, or 10 μl of sterile phosphate-buffered saline (PBS). This inoculation scheme resulted in an estimated delivered dose of 4×10^3 to 4×10^4 CFU to the dermis and epidermis of the skin (46). Inoculations were repeated at 14-day intervals (Figure 2. 1). Inoculations were carefully placed such that no two successive sites overlapped. In addition to the pigs that received all three inoculations, one pig received no inoculations, and two pigs received only the first inoculation.

Biopsy of samples.

Inoculation sites were excised 2 or 7 days following each inoculation with 6-mm-diameter skin punches (Acuderm, Ft. Lauderdale, Fla.). Tissues were bisected with sterile scalpels (Acuderm). One half of each biopsy was fixed in 4% paraformaldehyde in PBS,

while the other half was minced and plated on chocolate agar plates with 3 μg of vancomycin per ml for recovery and enumeration of viable *H. ducreyi*. If recovery plates were excessively contaminated, the recovery data were not included in our analyses. Fixed sample halves were embedded in paraffin, sectioned, stained with hematoxylin and eosin (Histopathology Reference Laboratory, Richmond, Calif.), and blindly scored for histological severity according to our previously developed scoring system (46) (data not shown).

Serum and complement collection.

Sera were collected weekly from all animals. One of the animals receiving a single inoculation suffered a leg injury and was euthanized prior to the completion of the study. This animal had serum collected for the final time on day 42 instead of day 49.

Collected blood was allowed to clot overnight at 4°C. Blood was centrifuged at room temperature in a bench top centrifuge at 408 × *g* for 10 min prior to the removal of serum. Serum was centrifuged again at room temperature in a bench top centrifuge at 408 × *g* for 10 min before aliquots were frozen at −80°C.

Pig complement was collected from separate naïve Yorkshire cross pigs. Blood was drawn from animals and clotted at 37°C for 30 min. Serum was removed and centrifuged at 4°C at 2,000 × *g* for 10 min. Serum was passed through a 0.45-μm syringe filter, aliquoted, and frozen immediately at −80°C. Aliquots were used only once as an active complement source.

Immune bactericidal assays.

H. ducreyi 35000HP was grown for 16.5 h from a freezer stock on chocolate agar at 35°C with 5% CO_2. Cells were harvested and suspended in 2 ml of BHI broth (Becton Dickinson, Cockeysville, Md.). Cells were vortexed for 5 s and allowed to settle for 5 min in order to remove large aggregates of *H. ducreyi*. After settling, the top 1 ml of the bacterial solution was removed and the cell density was adjusted such that the final concentration of bacteria was 100 to 500 CFU per 80 μl of media.

Bactericidal assays were performed as described (15, 16). Briefly, assays were performed in sterile 96-well plates (Falcon microtest tissue culture plate; Becton Dickinson, Franklin Lakes, N.J.). Each test well received 80 μl of cells and 10 μl (or 10%) of heat-inactivated test serum. Plates were incubated for 15 min at 35°C with 5% CO_2 after which 10 μl (or 10%) of either heat-inactivated or active fresh pig complement serum was added. Plates were mixed by tapping and then incubated for an additional 45 min. Bacteria were quantified by plating 60 μl from each well onto chocolate agar. Percent survival was determined for each immune serum sample tested by dividing the number of colonies that survived exposure to fresh serum complement by the number of colonies that survived with heat-inactivated serum complement and multiplying by 100. To ensure that the serum complement did not kill the bacteria in an antibody-independent fashion, we used BHI media in place of the serum antibody source in bactericidal assays. The average percent survival in the BHI medium was consistently

greater than 100% (data not shown), indicating that complement containing serum, in the absence of antibody, did not kill the bacteria. Samples were assayed in triplicate on three separate days.

Serum IgG determination.

Total serum immunoglobulin G (IgG) was measured following the manufacturer's protocol with a pig serum IgG enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, Tex.). *H. ducreyi*-specific IgG was measured by a similar method; however, instead of coating microplate wells (96-well MaxiSorp; Nalge Nunc, Rochester, N.Y.) with goat anti-pig IgG capture monoclonal antibody, wells were coated with whole-cell *H. ducreyi* lysate at a concentration of 10 μg/ml in 100 mM sodium carbonate, pH 9.6. The lysate was made by sonicating plate-grown *H. ducreyi* 35000HP on ice in a mixture of 50 mM sodium phosphate (monobasic) and 300 mM sodium chloride, pH 7.8. Insoluble particles and unbroken whole cells were removed from the sonicate mixture by centrifugation at 800 × *g* for 5 min. The sonicate was filtered (pore size, 0.2 μm) and assayed for total protein content (DC protein assay; Bio-Rad Laboratories, Hercules, Calif.). Aside from the coating, both ELISA formats were performed identically and in tandem. Coated wells were blocked with 1% bovine serum albumin in Tris-buffered saline (50 mM Tris, 0.15 M sodium chloride, pH 8), then washed with TBST (Tris-buffered saline plus 0.05% Tween 20). Sera were serially diluted twofold in TBST containing 1% bovine serum albumin, and a calibrator serum included

with the ELISA kit was run to assess the performance of each assay. Sera were incubated in the coated plates and washed with TBST before being incubated with a goat anti-pig IgG secondary antibody conjugated to horseradish peroxidase. After sera were washed again with TBST, tetramethyl benzidine detection substrate (KPL, Gaithersburg, Md.) was added to each well, allowed to develop, and stopped by the addition of 2 M H_2SO_4. The optical density at 450 nm (OD_{450}) was measured with a SpectraMax 340PC microplate spectrophotometer, and response curves were calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, Calif.). This software was also used to perform four-parameter curve fitting for the data. Titers were calculated by solving the four-parameter equation at an OD_{450} value of 15 times the assay background. The assay background was defined as the mean OD_{450} of all assay blank wells (containing all reagents except serum), plus three times the standard deviation. Response curves for general visual comparison were graphed with Microsoft Excel.

Passive transfer.

Pigs were infused with 25 ml of serum with a 30-ml syringe (Becton Dickinson, Franklin Lakes, N.J.) attached to a 6-in. male luer lock adapter extension set (Baxter Healthcare Corporation, Deerfield, Ill.) and an 18-gauge needle (Becton Dickinson, Franklin Lakes, N.J.). Two pigs were infused with normal pig serum, and five pigs were infused with serum from a repeatedly inoculated pig. One day after infusion, pigs were inoculated according to the procedure described above. Each pig was inoculated with live

bacteria at eight sites per ear, for a total of sixteen live inoculations, and the entire biopsy of each lesion was minced and plated for recovery. Day 7 recovery data from one pig infused with bactericidal serum were not included in the Results section due to overwhelming bacterial and fungal contamination on the recovery plates.

Statistical methods.

Statistical analysis was performed with Sigma Stat version 2.0 (Jandel Scientific, San Rafael, Calif.) and SAS software (SAS Institute Inc., Cary, North Carolina). Bacterial recovery counts were modeled with gamma regression modeling as a function of the day of the biopsy, and generalized estimation equations were used to adjust the data for random effects. The percentages of positive biopsies were analyzed via logistic regression analysis. This hierarchical model was used to accommodate the effect of collecting multiple biopsies from single animals. Data analysis with *t* tests was used to compare two groups for bactericidal assays, *H. ducreyi*-specific IgG titers, and recovery data from serum-infused animals.

Results

Recovery of viable bacteria decreased with repeated exposure to *H. ducreyi*.

Five pigs were inoculated with *H. ducreyi* three times at 2-week intervals (Fig. 2.1). Two and seven days following each inoculation, punch biopsies of inoculation sites were

collected and evaluated for lesion severity and recovery of live *H. ducreyi*. Biopsy results from both inoculation dilutions are presented together (Fig. 2.2), since analysis revealed that percentages of culture-positive lesion biopsies were identical for all time points (data not shown). All biopsies collected 2 days following the first and second inoculations contained live *H. ducreyi*, whereas only 55% of total biopsies taken 2 days after the third inoculation were culture positive. We were significantly more likely to recover bacteria from lesions biopsied 7 days after the first inoculation than after the third (89 versus 16% recovery; odds ratio, 45.33; 95% confidence interval, 4.89 to 420.48; $P = 0.0008$) (Fig. 2.2).

The average number of bacteria recovered from culture-positive lesions also dropped with repeated inoculations (Fig. 2.2). Gamma regression modeling revealed that significantly more bacteria were recovered after the first versus the third inoculation on both day 2 (odds ratio, 77.138; 95% confidence interval, 14.86 to 400.41; $P = <0.0001$) and day 7 (odds ratio, 310.00; 95% confidence interval, 61.30 to 1,567.75; $P = <0.0001$). Decreases in both the number of culture-positive lesions and in CFU recovered per lesion suggest that after three successive inoculations, pigs developed a modest but significant level of protection against *H. ducreyi* infection.

H. ducreyi-specific serum IgG levels increased over the course of multiple _H. ducreyi_ inoculations.

We measured total and *H. ducreyi*-specific serum IgG levels at three time points, preinoculation (day 0), 1 week after the second inoculation (day 21), and 2 weeks after the final inoculation (day 42).

H. ducreyi-specific serum IgG titers were greater in the day 42 sera of pigs inoculated three times than in the day 21 or day 0 sera of the same animals (Table 2.1). Day 42 sera from these pigs had 4.8-fold more *H. ducreyi*-specific IgG than sera collected from the same animals prior to inoculation (Table 2.1) ($P = 0.019$, *t* test). To determine if the elevated antibody titers in this group of pigs were due to multiple inoculations rather than to an age-dependent effect, we evaluated serum from an animal that was inoculated once on day 0. While there was an initial increase in *H. ducreyi*-specific IgG, this elevation did not persist over the course of the experiment (Table 2.1). This result indicated that the increase in *H. ducreyi*-specific IgG resulted from repeated exposure to *H. ducreyi* and not from a nonspecific change in the pigs' immune responses. There were no appreciable changes in the levels of total IgG in these sera over the course of the experiment (data not shown).

Serum from pigs inoculated multiple times displayed enhanced bactericidal activity against *H. ducreyi.*

We used an immune bactericidal assay to determine if the observed increases in *H. ducreyi*-specific antibody titers corresponded with increased bactericidal activity. We compared the bactericidal activity of preimmune sera (day 0), sera collected 2 weeks after the first inoculation (day 14), 2 weeks after the second inoculation (day 28), and 3 weeks after the third inoculation (day 49) (Fig. 2.1). We also tested sera collected from one uninoculated pig and two pigs that received only one inoculation.

The average percent survival of *H. ducreyi* in preimmune sera was 95% ± 35%, indicating that none of the preimmune serum samples (n = 8) exhibited bactericidal activity.

The average percent survival of *H. ducreyi* in day 14 sera of animals receiving only one inoculation was 35% ± 32% (n = 7). The average percent survival of bacteria in day 28 sera from twice-inoculated animals was 11% ± 17%. In sera drawn 3 weeks after the third and final inoculation, the average percent survival of *H. ducreyi* was 23% ± 10% (Fig. 2.3). The average percent survival of *H. ducreyi* in sera from the uninoculated pig was approximately 100% regardless of the day the serum was drawn (Fig. 2.3). This result indicated that emergence of bactericidal activity was not an age-related phenomenon.

We also wanted to determine if this enhanced bactericidal activity resulted from just the first inoculation or was dependent upon the multiple inoculation protocol. While day 14 sera of animals receiving a single inoculation displayed enhanced bactericidal activity, the average percent survival of *H. ducreyi* in sera drawn 4 weeks after a single inoculation was 81 ± 48%. A similar 77 ± 35% of bacteria survived in sera collected 7 weeks after a single inoculation (Fig. 2.3). In fact, sera drawn at the end of the study from pigs that had received a single inoculation did not exhibit a statistically significant difference in bactericidal activity compared to activity in preimmune serum. This finding suggested that while one inoculation with *H. ducreyi* increased bactericidal antibody titers, this enhancement was transient in the absence of repeated inoculations.

Day 49 sera from pigs inoculated three times had statistically greater bactericidal activity than all preimmune sera ($P = <0.001$, *t* test). Day 49 sera from this group of pigs also had much greater bactericidal activity than day 49 sera from animals that received one inoculation or that remained uninoculated (Fig. 2.3). The increased bactericidal activity of sera collected from the animals receiving three inoculations corresponded with total *H. ducreyi*-specific IgG titers as measured by ELISA.

The passive transfer of bactericidal serum protected naïve animals against *H. ducreyi* challenge.

We hypothesized that elevated titers of *H. ducreyi*-specific antibodies, possibly through the action of complement-mediated killing, were providing the animals that received repeated inoculations with protection against *H. ducreyi* challenge. To test this idea, we transfused immune serum from a pig that received multiple inoculations into naïve pigs before *H. ducreyi* challenge. As a control, we also challenged naïve pigs infused with serum from an uninoculated pig (normal pig serum). All lesions collected from pigs infused with normal pig serum contained viable bacteria. Two days after inoculation, 82.5% of the biopsies collected from all immune serum-infused animals contained viable bacteria. Seven days after inoculation, this percentage dropped to 20%. Two of the pigs infused with immune serum yielded exclusively sterile lesions on day 7. Only 25 and 43% of day 7 lesions from the other two animals infused with immune serum contained viable bacteria (Fig. 2.4). The difference in the percentages of *H. ducreyi*-positive biopsies per

pig for animals infused with the immune versus normal pig serum was statistically significant for day 7 biopsies ($P = 0.006$, *t* test).

Discussion

After three successive experimental chancroid infections, pigs developed a modestly protective immune response against *H. ducreyi*. The recovery of viable organisms from lesion biopsies as well as the number of culture-positive lesions decreased throughout the experiment. This decreased recovery corresponded directly with increased serum levels of *H. ducreyi*-specific IgG and enhanced bactericidal activity. This finding suggested that the humoral component of the immune response was involved in mediating protection against *H. ducreyi*. Because naïve pigs infused with serum from a pig that received repeated inoculations were protected against *H. ducreyi* challenge, we concluded that the humoral component of the immune response was playing a major role in mediating protection.

The successful transfer of protection along with the transfer of immune serum distinguishes our findings from similar experiments performed with the temperature-dependent rabbit model of chancroid (40). In the temperature-dependent rabbit model, a single previous experimental infection with *H. ducreyi* (26), immunization with cell wall components (26), a pilus preparation (11, 12), or purified hemolysin (14) protected rabbits against future experimental challenge. However, passive transfer of whole-cell *H.*

ducreyi-specific or pilus-specific IgG fractions did not confer protection (12). Passive transfer was not successful despite the fact that passively immunized rabbits displayed sustained, titratable antibody levels throughout the experiment (12). Perhaps this result was due to the fact that immune rabbit sera do not possess bactericidal activity against *H. ducreyi* (20, 28).

The protection observed in the pig model may initially appear to contradict conclusions drawn from the human challenge model of chancroid. Previous experimental infection neither prohibits nor inhibits the development of a second experimental infection in human volunteers (4, 49). However, careful comparison of recovery data from the human and swine models of chancroid revealed similarities. Human challenge studies with a single infection reported that 57 to 100% of lesion biopsies contain viable *H. ducreyi* (2, 3, 7, 8, 19, 39, 48, 50, 53, 54). In a human challenge reinfection study, viable *H. ducreyi* was recovered from 83% of biopsies from previously infected people and 67% of biopsies from individuals infected for the first time (4). These percentages are consistent with the percentages of culture-positive biopsies observed in pigs after the first and second inoculations. Two and seven days after the first inoculation, 100 and 89% of the swine biopsies were culture positive, respectively. All 20 of the biopsies collected 2 days after the second inoculation were positive, and 42% of the biopsies collected 7 days after the second inoculation were also culture positive. The significant drops in both the recovery and the number of culture-positive lesions did not occur until after the third inoculation.

It was concluded that experimental human infection to the pustular stage of disease did not protect people against a subsequent chancroid infection (4) because a naïve control group and a group of individuals that had been previously experimentally infected exhibited equivalent abilities to form both pustules and papules. However, after a single previous inoculation, pigs also developed papules and pustules in response to inoculation with *H. ducreyi*. Pig lesion histology scores dropped significantly only after the third inoculation (data not shown). After the third inoculation, the average histology scores for the PBS and live inoculations and the heat-killed bacteria inoculations were virtually identical. While we saw the development of protective immunity in the swine model of chancroid infection, the results from the first two rounds of pig inoculations are similar to results from the human challenge model of chancroid infection.

Pigs developed antibodies to *H. ducreyi* after single (29) and repeated inoculations. In contrast, initial (38, 48) and repeated (4) experimental human infection up to 14 days or the pustular stage does not evoke an antibody response to bacterial proteins or lipooligosaccharides. Unlike experimental infection, naturally occurring chancroid results in the development of a humoral response to *H. ducreyi* (1, 9, 10, 17, 36, 42, 47). Once chancroid develops, the likelihood of producing *H. ducreyi*-specific antibodies increases along with ulcer duration (10, 42). Patients with genital ulcers persisting in excess of 4 weeks have the strongest humoral response (10).

Antibodies may mediate protection through enhanced opsonization, increased bactericidal activity, blockage of attachment, or some combination of all three effects.

While people with naturally occurring chancroid infection develop antibodies against *H. ducreyi*, these antibodies are not bactericidal (20). In contrast, antibodies produced by pigs that received three inoculations were bactericidal against *H. ducreyi*, and serum containing these antibodies conferred enhanced bacterial clearance when transferred to naïve animals. This outcome strongly suggested that the bactericidal ability of the *H. ducreyi*-specific swine antibodies provided protection against chancroid.

The development of bactericidal activity in pigs was specifically dependent on the multiple inoculation protocol as the uninoculated animal did not display enhanced bactericidal ability and animals receiving single inoculations displayed only transient increases in bactericidal activity. This greatly enhanced bactericidal activity was accomplished with only a fivefold increase in *H. ducreyi*-specific antibody titer. While this increase is not a large elevation in titer, protective antibody titers following natural infections are often lower than the titers seen postvaccination. For example, both previous natural infection and vaccination against hepatitis B infection are protective, but after natural seroconversion, the average geometric mean titers of anti-hepatitis B antibodies are 41-fold lower than titers at the peak of response in vaccinees (24).

We examined the longevity of the protection seen in the three-times inoculated animals by inoculating a single pig from this group a fourth time with *H. ducreyi* 70 days after the third inoculation. While 25% of the lesions biopsied 2 days after the fourth inoculation yielded live organisms, none of the lesions biopsied 7 days after the fourth inoculation yielded live organisms (data not shown). Reduced bacterial recovery once

again corresponded with increased bactericidal ability as 66 ± 18% of *H. ducreyi* cells survived in serum drawn just prior to the fourth inoculation, while only 15 ± 4% of the cells survived in serum drawn 7 days after the fourth inoculation (data not shown). The rapid development of bactericidal activity suggests the existence of antigen-specific memory B cells and implies that memory B cell generation will be an important component of any successful human *H. ducreyi* vaccine.

We have begun to identify targets of swine antibodies produced in response to repeated *H. ducreyi* exposure. All pigs receiving repeated inoculations appear to develop antibodies to the same distinct set of *H. ducreyi* antigens (data not shown). We hope to identify both the specific antigens and their roles as bactericidal antibody targets. The comparison of proteins eliciting antibody responses could also be important in understanding how a humoral response is protective in pigs but not in people.

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We are grateful for the technical assistance of Patty Routh; her expertise with pigs enables the swine model of chancroid to continue. We also thank Drew Reinbold for his technical assistance.

Attributions

Leah Cole, Kristen Toffer, Lani San Mateo and I inoculated pigs and analyzed lesion biopsies obtained from them. Lani San Mateo began development of the ELISA for *H. ducreyi*-specific pig serum antibodies; I optimized the assay and analyzed pig serum samples to generate the antibody titer data presented in this paper. Leah Cole performed the bactericidal assays with assistance from Kristen Toffer and Drew Reinbold. Leah Cole and I performed the passive infusion experiments with assistance from Drew Reinbold. This work was published in the journal Infection and Immunity, Volume 71, pages 6971-6977. Permission has been granted to reprint this material.

Table 2.1. Summary of *H. ducreyi*-specific IgG titers from an uninoculated pig and from pigs inoculated one and three times

Treatment (n)[a]	Titer $^{-1}$ (SD)		
	Day 0[b]	Day 21[c]	Day 42[d]
Three inoculations (4/5)	54 (3.2)	190 (1.8)[e]	260 (1.8)
One inoculation (1/2)	24	56	25
Uninoculated (1/1)	120 (1.1)	65 (1.0)	50 (1.0)

[a] *n*, number of pigs assayed/number of pigs treated

[b] Day 0, preimmune serum

[c] Day 21 serum was collected at the time corresponding to 1 week after the second set of inoculations (See Figure 2.1).

[d] Day 42 serum was collected 2 weeks after the time corresponding to the third inoculation.

[e] Only two pigs were assayed at day 21.

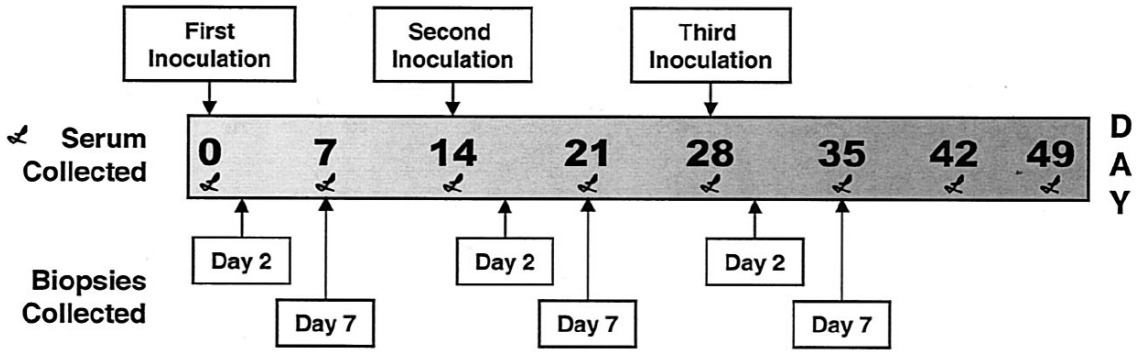

Figure 2.1. Timeline of repeat inoculation study with Yorkshire cross pigs. Inoculations occurred at 14-day intervals. The ears of the pigs were inoculated with live *H. ducreyi* at four sites per ear per inoculation. Lesion biopsies were collected either 2 or 7 days after each inoculation. Five pigs were inoculated three times, two pigs were inoculated once, and one pig was never inoculated. Serum was collected from all pigs on a weekly basis.

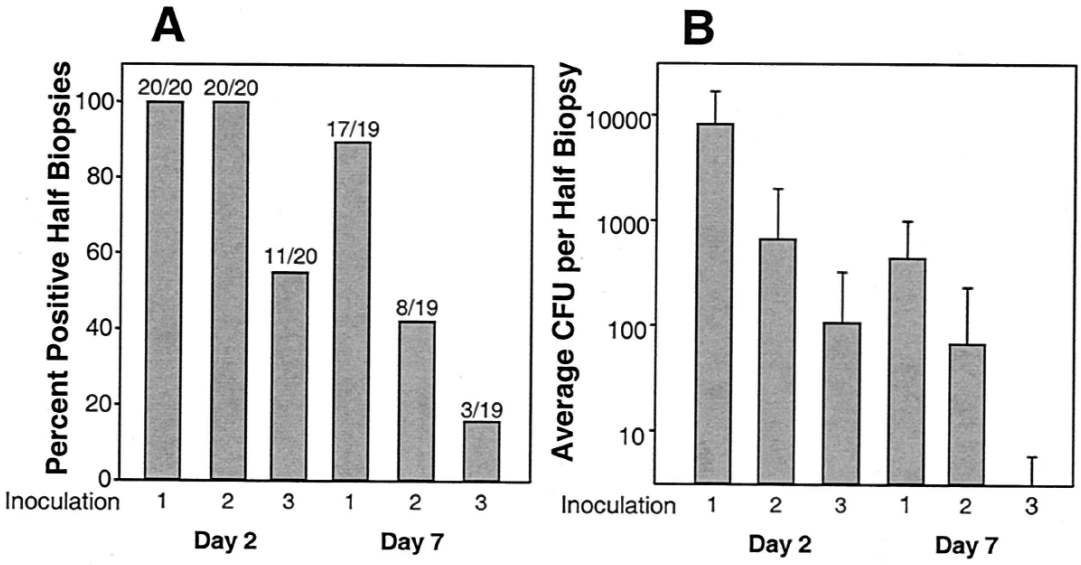

Figure 2.2. Percentage of all half-biopsies which were positive for recovery (A) and average recovery of CFU per half-biopsy (B).
Data are from five pigs inoculated three times at 14-day intervals. Four lesions from live *H. ducreyi* inoculations were collected from each animal 2 and 7 days after each inoculation. CFU per half-biopsy were enumerated by mincing and plating half of each biopsy on chocolate agar. Recovery of one bacterium was sufficient to mark the lesion biopsy as positive for recovery. (A) Fraction numerators are the number of lesion biopsies positive for recovery, whereas denominators are the total number of lesion biopsies analyzed at that time point. Bars represent percentages of culture-positive lesion biopsies. (B) Average CFU per half-biopsy was calculated for each time point by dividing the total CFU recovered by the number of biopsies analyzed. This graph displays means and standard deviations.

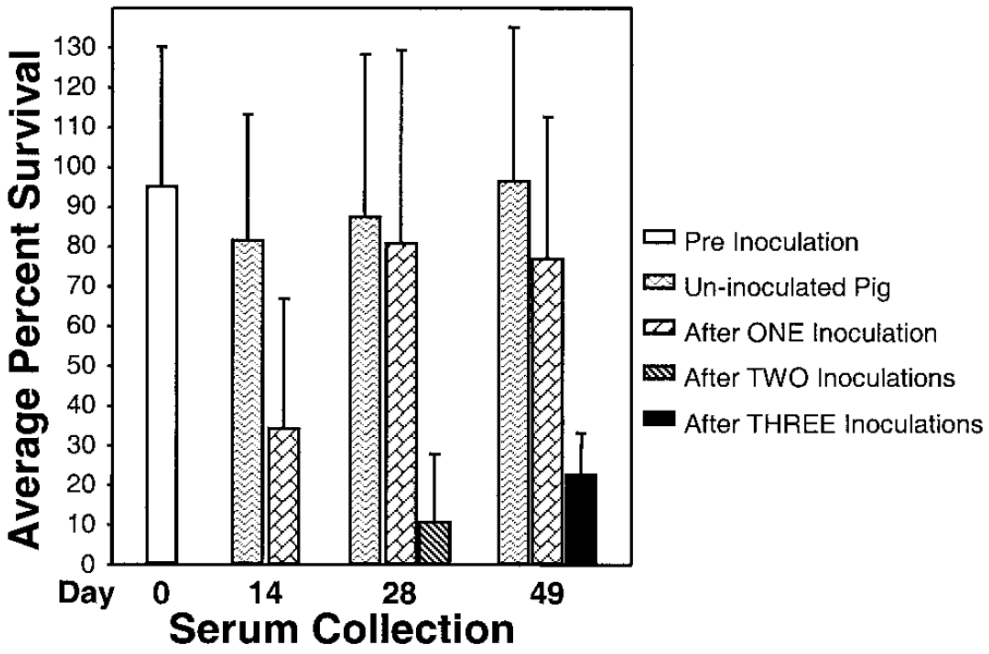

Figure 2.3. Average percent survival of *H. ducreyi* in immune bactericidal assays with heat-inactivated pig sera collected after three, two, one, or zero inoculations with *H. ducreyi*.

Bars represent average percent survival of *H. ducreyi* in sera drawn on the day indicated after treatment described. The day 0 data bar represents survival of *H. ducreyi* in sera drawn from all eight animals prior to any inoculations. Day 14 data bars represent assays done with serum drawn from an uninoculated animal and sera drawn from seven pigs that had received one inoculation on day 0. Day 28 data bars represent assays done with serum drawn from one uninoculated pig, sera drawn from two pigs that received only one inoculation on day 0, and sera drawn from five pigs that had received two prior inoculations on day 0 and day 14. Day 49 data bars represent the percent survival of *H. ducreyi* in sera drawn on day 49 from three groups of animals: a single uninoculated pig, one pig that had been inoculated once on day 0, and five pigs that were inoculated three times on days 0, 14, and 28. Error bars illustrate standard deviations. Data were subjected to an analysis of variance.

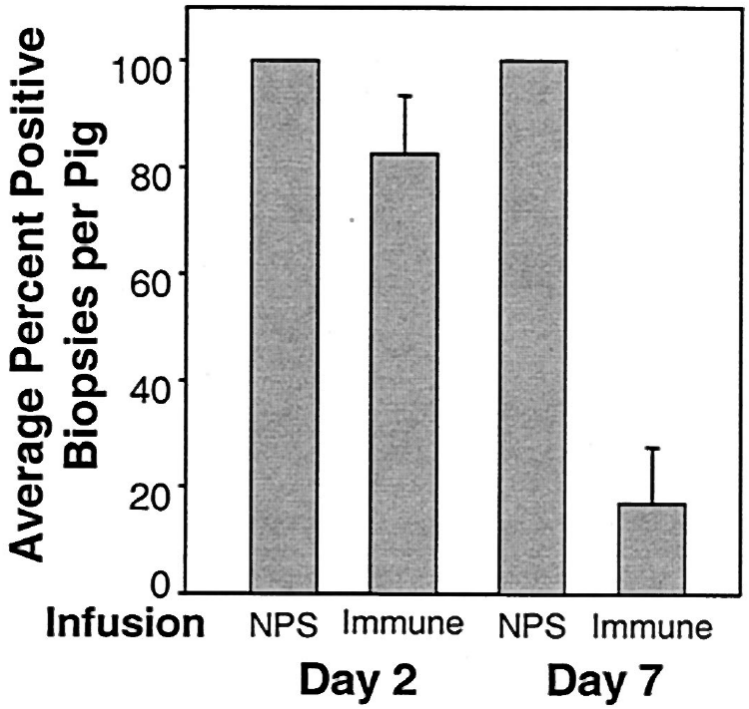

Figure 2.4. Average percentages of *H. ducreyi*-positive biopsies per pig after passive transfer of immune or normal pig serum (NPS) to naïve animals.
Pigs were inoculated with *H. ducreyi* 1 day after infusion with either NPS or immune serum. Lesion biopsies were collected either 2 or 7 days after inoculation. Entire lesion biopsies were minced and plated for recovery. Bars represent the average percentage of culture-positive biopsies per pig. Error bars represent standard errors of the means. Analysis via *t* test revealed that while there was not a statistically significant difference in the percentage of culture-positive biopsies per pig on day 2, there was on day 7 ($P = 0.006$).

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CHAPTER 3

Expression of *Haemophilus ducreyi* Collagen Binding Outer Membrane Protein NcaA Is Required for Virulence in Swine and Human Challenge Models of Chancroid

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Haemophilus ducreyi, the etiologic agent of the sexually transmitted genital ulcer disease chancroid, has been shown to associate with dermal collagen fibers within infected skin lesions. Herein we describe NcaA, a previously uncharacterized outer membrane protein that is important for *H. ducreyi* collagen binding and host colonization. An *H. ducreyi* strain lacking the *ncaA* gene was impaired for adherence to type I collagen but not fibronectin (plasma or cellular forms) or heparin. The mutation had no effect on serum resistance or binding to HaCaT keratinocytes or human foreskin fibroblasts *in vitro*. *E. coli* expressing *H. ducreyi* NcaA bound to type I collagen, demonstrating that NcaA is sufficient to confer collagen attachment. The importance of NcaA in *H. ducreyi* pathogenesis was assessed using both swine and human experimental

models of chancroid. In the swine model, 20% of lesions from sites inoculated with the *ncaA* mutant were culture-positive for *H. ducreyi* 7 days after inoculation compared to 73% of wild type-inoculated sites. The average number of CFU recovered from mutant-inoculated lesions was also significantly reduced compared to wild type at both 2 and 7 days after inoculation. In the human challenge model, 8 of 30 sites inoculated with wild type *H. ducreyi* progressed to the pustular stage, compared to 0 of 30 sites inoculated with the *ncaA* mutant. Together these results demonstrate that collagen binding protein NcaA is required for *H. ducreyi* infection.

Introduction

Haemophilus ducreyi is the etiologic agent of the sexually transmitted genital ulcer disease (GUD) chancroid. Chancroid is rare in the United States and Western Europe. It is prevalent in tropical resource-poor regions and is endemic in certain areas of Africa and Asia (11, 38, 39, 47), where there is also a significant confluence of chancroid infection and HIV-1 seropositivity (17, 30, 40). In these regions, case control studies have shown that the relative risk of acquiring HIV infection for GUD patients ranged from odds ratios of 3 to 18.2 (20, 49, 60). Per individual sexual act, GUD produces a 4- to 23-fold enhancement of HIV acquisition (22, 33), and GUD in an HIV-positive person doubles the relative risk per coital act of viral transmission to an HIV-negative partner (22, 61). Furthermore, HIV-positive chancroid patients often present ulcers that are more severe, longer-lasting, and more difficult to cure (31, 45). These observations

outline the strong synergistic relationship between GUD and HIV (23, 24). Aggressive control of chancroid, a prevalent and highly transmissible GUD, may have the potential to curtail the global spread of HIV (34, 37, 42).

Chancroid presents as a painful genital ulcer with a ragged erythematous border. This lesion emits a purulent exudate replete with macrophages, neutrophils, T and B lymphocytes and bacteria (both *H. ducreyi* and other opportunistic species) (32). *H. ducreyi* does not cause disseminated infection in healthy or immunocompromised individuals, and appears to be well-adapted to survive in the skin of its obligate human host (59). However, the mechanism by which *H. ducreyi* colonizes skin remains poorly understood. *H. ducreyi* attaches to the HaCaT keratinocyte cell line *in vitro* (62), and this association is dependent upon expression of the outer membrane protein DsrA (15). DsrA also confers resistance to complement-mediated killing by normal human serum (19). *H. ducreyi* lacking DsrA is attenuated in both human and swine experimental chancroid models (12), but it is not clear how the keratinocyte attachment or serum resistance properties of DsrA specifically contribute to this phenotype. *H. ducreyi* also adheres to human foreskin dermal fibroblasts and forms microcolonies *in vitro*; this aggregative-adhesive phenotype depends on expression of the fimbria-like protein encoded by the *flp* gene cluster (41). Human volunteers inoculated with an *flp* mutant display lesions that fail to progress from papular to pustular stage, and live *H. ducreyi* was recovered from only 1 of 31 of these sites (53), suggesting that the above phenotype contributes to *H. ducreyi* survival in skin.

Despite the observation that *H. ducreyi* adheres to skin epithelial cells and fibroblasts *in vitro*, direct association between *H. ducreyi* and these types of cells within the chancroid lesion has not been proven. *H. ducreyi* was, however, shown to bind the extracellular matrix (ECM) proteins fibronectin, laminin and collagen types I and III *in vitro* (9). In early-stage lesions derived from the human challenge model, *H. ducreyi* colocalized with macrophages and neutrophils within the pustule and also associated with fibrin and collagen in the dermis directly beneath the pustule (8). These findings suggest that adherence to ECM proteins, which comprise a major structural component of skin, may be an important contributing factor in *H. ducreyi* skin colonization.

We recently found that protection against *H. ducreyi* infection in the swine model of chancroid coincides with the emergence of *H. ducreyi*-specific bactericidal serum antibodies, and that transfer of this immune serum protects naïve pigs against *H. ducreyi* challenge (16). Antibodies present in this immune serum recognized a distinct subset of *H. ducreyi* outer membrane proteins. Herein we describe one of these proteins, termed NcaA, and its role in mediating binding of *H. ducreyi* to collagen binding and in *H. ducreyi* pathogenesis.

Materials and Methods

Bacterial Growth Conditions.

H. ducreyi strains were grown on chocolate agar containing 1.5% Bacto agar, 2.5%

brain heart infusion (BHI), 1% bovine hemoglobin, 1% IsoVitaleX (Becton Dickinson, Cockeysville, MD), 5% newborn calf serum and 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD) at 35°C in a humidified incubator with 5% CO_2. *E. coli* strains were propagated in LB broth or agar at 37°C. Ampicillin, kanamycin, vancomycin, or chloramphenicol was added to media when indicated to a final concentration of 100, 40, 3, or 1 μg/ml respectively.

Radioimmunoprecipitation of *H. ducreyi* outer membrane proteins.

H. ducreyi 35000HP was grown overnight on chocolate agar as a confluent lawn, harvested, and resuspended in PBS. This suspension was added to a precoated IODO-GEN tube (Pierce Biotechnology, Rockford, IL) with 1.0 mCi of ^{125}NaI. Labeling was quenched after 3 min with an excess of GCB broth. Surface-iodinated bacteria were washed and mixed with preimmune or *H. ducreyi*-immune pig serum, incubated 15 min at RT with occasional mixing, again washed with PBS and pelleted. The resulting cell pellet was then resuspended in RIP buffer (2% Zwittergent 3-14, 50 mM Tris pH 8.0, 5 mM EDTA, 0.15 M NaCl) and incubated with rocking for 30 min at 37°C. Insoluble matter was removed by centrifugation (5 min at 14,000 x *g*), and the supernatant containing solubilized antibody-labeled outer membranes was mixed with protein G agarose (Sigma Chemical, St. Louis, MO). After overnight rocking at 4°C, the slurry was washed 3 times with RIP buffer and once with diH_2O. The resulting pellet was resuspended in Laemmli sample buffer containing β-mercaptoethanol, boiled, and

resolved by SDS-polyacrylamide gel electrophoresis. Resolved bands of iodinated (outer membrane) proteins bound by preimmune or *H. ducreyi*-immune pig serum were visualized by autoradiography.

Identification, cloning, and mutagenesis of *ncaA*.

H. ducreyi outer membrane proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Protein bands were excised and subjected to Edman degradation by the UNC Protein Sequencing & Peptide Synthesis Facility (University of North Carolina, Chapel Hill, NC). Amino acid sequences were compared using EMBOSS pairwise alignment algorithms hosted by the European Bioinformatics Institute. (http://www.ebi.ac.uk/emboss/align/index.html). The *ncaA* gene was amplified by PCR from *H. ducreyi* 35000HP genomic DNA using forward primer $^{5'}$CTAGGCTAATGAGAGGTATATCG$^{3'}$ and reverse primer $^{5'}$TTGTACGCATCGCTTGTTCC$^{3'}$. The resulting product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and was verified by automated DNA sequencing (UNC Genome Analysis Facility, Chapel Hill, NC). To produce an inactivated allele of *ncaA*, a 134 bp segment of *ncaA* between two *Nde* I sites was excised and replaced with a chloramphenicol acetyltransferase (*cat*) cassette derived from pUNCH40 (57). The *cat*-interrupted *ncaA* gene (*ncaA*::*cat*) was subcloned into plasmid pRSM2072 (gift of Robert S. Munson, Jr.) and the resulting construct was used as a suicide vector to perform allelic

exchange in *H. ducreyi* as described by Bozue *et al.* (14). *H. ducreyi* 35000HP was transformed with pRSM2072-*ncaA*::*cat* and plated on chloramphenicol to select cointegrates. Cointegrates were then plated on media containing chloramphenicol and X-gal (40 μg/ml) to counterselect for excision of the pRSM2072 vector backbone and the wild type *ncaA* allele. Southern blot and PCR analysis of the mutant confirmed that a single copy of the *cat* cassette had been inserted into *ncaA* (data not shown). Transcription of genes adjacent to the *ncaA* locus (Fig. 3.1) in 35000HP and 35000HP*ncaA* was found equivalent by reverse transcriptase PCR, showing that *ncaA* inactivation did not exert a negative polar effect on local transcription. To examine whether 35000HP*ncaA* had phenotypes other than that caused by the mutation, 35000HP*ncaA* was compared to the master 35000HP stock used to infect human volunteers. The phenotypes included comparisons of growth rates in broth, OMP and LOS profiles, as previously described (21). 35000HP and 35000HP*ncaA* had similar generation times in broth (data not shown). OMP and LOS from 35000HP and 35000HP*ncaA* were analyzed by SDS-PAGE, followed by Coomassie blue and silver staining. The OMP and LOS profiles demonstrated no difference between the parent and the mutant (data not shown). Because NcaA is a low abundance protein as determined by Coomassie blue staining, ablation of NcaA protein expression in the mutant strain was confirmed by Western blot analysis with *H. ducreyi*-immune pig serum (Fig. 3.2). Whole-cell lysates of 35000HP and 35000HP*ncaA* were heated in sample buffer containing SDS and β-mercaptoethanol, then resolved by SDS-PAGE and blotted to

nitrocellulose. The blot was probed with *H. ducreyi*-immune pig serum (1:1000) followed by peroxidase-conjugated rabbit-anti pig Ig (1:32,000; Sigma Chemical, St. Louis, MO) and chemiluminescent detection (SuperSignal Pico, Pierce Biotechnology, Rockford, IL).

Serum Susceptibility Assay.

Serum susceptibility assays were performed as previously described (19). Briefly, bacteria grown on solid media for 16-18 hours were collected and suspended in 2 ml of BHI, vortexed for 5 seconds and allowed to settle for 5 minutes. After settling, the upper 1 ml was removed and the cell density was adjusted such that the final concentration of bacteria was approximately 1-5 x 10^3 CFU/ml. Bacteria were mixed with fresh or heat-inactivated normal swine serum to a final concentration of 25% or 50%. Following incubation for 45 minutes (35°C, 5% CO_2) the bacterial suspensions were plated to count viable CFU. Percent survival was calculated by dividing the number of CFU from fresh serum by the number from heat-inactivated serum and multiplying by 100. Assays were performed in triplicate and on three separate days.

Fluorescein labeling of bacterial strains.

Overnight cultures of *H. ducreyi* grown on chocolate agar or *E. coli* grown in LB broth were collected and washed three times in phosphate-buffered saline (PBS) followed by passage through a 30-gauge needle to disperse large aggregates. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes, Eugene, OR) in DMSO was

added to a final concentration of 12 μM and the suspension was incubated with gentle mixing at 35°C for 25 minutes. *H. ducreyi* labeling reactions were quenched with an equal volume of BHI media supplemented with 1% IsoVitaleX (Becton Dickinson, Cockeysville, MD), 1% hemoglobin, (Becton Dickinson, Cockeysville, MD), and 10% fetal bovine serum (Life Technologies, Rockville, MD). *E. coli* labeling reactions were quenched by addition of BSA to a final concentration of 1%. The resulting fluorescein-labeled bacteria were collected by centrifugation at 1040 x *g* for 10 minutes and resuspended in LB (*E. coli*) or in BHI supplemented with 1% IsoVitaleX and 1% hemoglobin (*H. ducreyi*). Bacteria were then incubated for 40-60 minutes at 35°C with shaking. The doubling times of fluorescein-labeled and unlabeled *H. ducreyi* were similar (data not shown).

Extracellular Matrix Protein Attachment Assay.

White 96-well plates (Fluoronunc, Nalge Nunc, Rochester, NY) were coated with bovine or human type I collagen (Chondrex, Redmond, WA), human cellular fibronectin or human plasma fibronectin (Sigma-Aldrich, St. Louis, MO) diluted in collagen dilution buffer (Chondrex, Redmond, WA) or 50 mM sodium carbonate pH 9.6 (fibronectins). Similar results were obtained in binding assays using bovine- and human-derived type I collagens. Plates were incubated overnight at 35°C (fibronectin) or 4°C (collagen), washed three times with PBS containing 0.05% Tween-20 (PBST), and blocked with 300 μl of 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS for two hours at

35°C. Wells were washed three times in PBS. Fluorescein-labeled or non-labeled bacteria in BHI (200 µl) were added to appropriate wells. Separate aliquots of the bacterial suspensions were serially diluted and plated to determine CFU input. After 4 hours at 35°C the wells were washed three times with PBST and 200 µl of PBS was added to all wells prior to reading fluorescence. Fluorescence emission at 520 nm was measured following excitation at 490 nm using a microplate spectrometer (Model LS50B, Perkin Elmer Ltd., Beconsfield, Buckinghamshire, England). Standard curves were established for each strain in each assay to allow calculation of attached CFU from fluorescence intensity.

Animal Care.

Pigs were housed in an American Associated for Accreditation of Laboratory Animal Care-accredited P2 containment facility at North Carolina State University College of Veterinary Medicine. Animals were sedated for all procedures with 0.3 ml per 22.7 kg body weight of an anesthetic formulation consisting of: Telazol (tiletamine HCl, 50 mg/ml and zolazepam HCl, 50 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA), 50 mg/ml ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 50 mg/ml xylazine (Miles Laboratories, Shawnee Mission, KS). Atropine sulfate (Phoenix Scientific Inc., Joseph, MO) was given at 0.5 to 1 ml to slow salivary and bronchial secretion. Pigs were maintained in the same room but in separate enclosures during the course of the study.

Virulence Testing in Pigs.

Inoculum preparation, inoculation and biopsy procedures were previously described (27, 50). Briefly, *H. ducreyi* grown on chocolate agar was suspended in PBS and passed through a 30-gauge needle to disperse aggregates. Multi-Test skin test applicators (Lincoln Diagnostics, Decatur Ill.) were loaded with 10 μl of inoculum and pressed into the dorsal side of the ear. Each round of inoculations entailed 4 to 6 application sites per ear per strain tested. Biopsies of experimental lesions collected two and seven days after inoculation using 6 mm skin biopsy punches (Acuderm, Ft. Lauderdale, FL) were minced and plated on chocolate agar containing vancomycin to detect viable *H. ducreyi* within lesions.

Virulence Testing in Human Volunteers.

Healthy adult male and female volunteers over 18 years of age were recruited for the study. Subjects gave informed consent for participation and for HIV serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University-Purdue University of Indianapolis. The experimental challenge protocol, preparation and inoculation of the bacteria, calculation of the estimated delivered dose (EDD), surface cultures and clinical observations were done exactly as described previously (4, 5, 54, 55, 58). Each subject was inoculated on one arm with 3 identical doses of the parent and on the other arm with 3 doses of twofold serial dilutions of the mutant. Subjects were

observed until they reached a clinical endpoint, defined as either 14 days after inoculation, development of a pustule that was either painful or greater than 4 mm in diameter, or resolution of infection at all sites. Once a clinical endpoint was achieved, the code was broken, and sites with clinical disease, if present, were biopsied. The subjects were then treated with two doses of oral ciprofloxacin (5, 55).

To confirm that the inocula were correct and that no phenotypic changes occurred during infection, individual colonies from the inocula, surface cultures, and biopsy specimens were picked, suspended in freezing medium, and frozen in 96-well plates. The colonies were scored for susceptibility to chloramphenicol. If available, sufficient colonies ($n \geq 30$) from an individual specimen were scored so that there was a 95% probability that $\leq$11% of the colonies would have the incorrect phenotype (1).

Statistical Analysis.

Data from the pig model were analyzed using SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA) and SAS version 9 (SAS Institute Inc., Cary, NC). Numbers of viable wild type and mutant *H. ducreyi* CFU recovered from lesion biopsies were analyzed using Poisson regression. Percentages of biopsies culture-positive were analyzed via logistic regression modeling of probabilities of recovery. Both regression models were adjusted for variability among pigs using generalized estimating equations (GEE). Comparisons of papule and pustule formation rates between the two strains in the human challenge model were performed using a logistic regression model using GEE to account

for the correlation among sites within the same subject, as described (53). The GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals for these rates. When a rate was zero, the exact binomial confidence intervals were calculated based on the number of subjects rather than the number of sites. The *z*-significance test of two means was used to compare the adherence of different strains of bacteria to type I collagen (Fig. 3), and the *t*-test was used to determine the significance of NcaA-expressing *E. coli* bound to collagen (Fig. 4).

Results

Identification of a putative oligomeric coiled adhesin in *H. ducreyi* .

We recently demonstrated that pigs repeatedly inoculated with *H. ducreyi* develop a modest antibody-mediated protective response against reinfection (16). Targets of this antibody response were identified by immunoprecipitation of ^{125}I-labeled *H. ducreyi* outer membrane proteins (OMPs) using immune sera from multiply-inoculated pigs. Seven distinct *H. ducreyi* outer membrane proteins were precipitated exclusively by immune sera (data not shown). One of these proteins with an apparent molecular weight of >100 kDa was isolated and identified via N-terminal amino acid sequencing and comparison to the published *H. ducreyi* genome sequence. The N-terminal sequence, I T T E S I P T, matched predicted amino acids 21-28 of a previously uncharacterized *H. ducreyi* OMP

(hypothetical protein HD1920 BLAST accession NP_874254) with a predicted molecular weight of ~33 kDa. We named this protein NcaA.

The NcaA amino acid sequence is similar to the *H. ducreyi* OMP DsrA (25.1% identity and 42.6% similarity), *Yersinia enterocolitica* YadA (23.0% identity and 39.9% similarity), *Moraxella catarrhalis* UspA proteins (21.0-22.0% identity and 35.6-39.3% similarity), and the *Escherichia coli* Eib proteins (20.7-27.2% identity and 38.0-43.2% similarity). These proteins have been described as oligomeric coiled adhesins (Oca) (48), a subfamily of the type Vc surface-attached oligomeric autotransporter family (25). They are typified by a signature C-terminal 9 amino acid stretch of alternating hydrophobic amino acids terminating with phenylalanine or tryptophan which comprises an outer membrane localization motif. The C-terminal region of these proteins also appears to mediate the formation of multimers that can remain stable even when heated with detergent and reducing agents (48). The disparity between the hypothetical molecular weight of monomeric NcaA and its dramatically slower migration in SDS-PAGE (Fig. 2) is consistent with similar proteins of this nature. The sequence of the 79 C-terminal amino acids of NcaA is 98% similar to the consensus Oca sequence and also possesses the 9 amino acid terminus motif. Oca proteins, in addition to the C-terminal signature sequence, share other properties including involvement in adherence, binding of extracellular matrix (ECM) proteins, and resistance to complement-mediated killing. These common functions led us to investigate whether NcaA mediates adherence to eukaryotic cells and ECM proteins.

Extracellular Matrix Protein Binding Properties of NcaA.

To determine whether NcaA binds ECM proteins we compared the ECM-binding properties of wild type *H. ducreyi* 35000HP with a mutant unable to express NcaA due to insertion-deletion inactivation of the *ncaA* gene (Fig. 3.2). Fluorescein-labeled 35000HP and the *ncaA* mutant (35000HP*ncaA*) were incubated in ECM-coated microplate wells. Assays were performed by incubating a bacterial suspension of constant density in wells coated with a range of ECM concentrations from 0 to 80 μg/ml. A minimum *H. ducreyi* density of 10^7 CFU/ml was sufficient to ensure that binding saturation was achieved at the highest ECM concentrations. Bacterial binding was determined by measuring the fluorescence intensity of each well following incubation and washing, then relating that intensity to an assay-specific standard curve correlating fluorescence to CFU.

Binding of the *ncaA* mutant strain to type I human collagen was significantly less than that of its isogenic parent strain *H. ducreyi* 35000HP (Fig. 3.3) with statistically significant differences in attachment at all ECM concentrations tested except 0.002 μg/ml. In contrast, there was no difference in the level of binding of 35000HP or 35000HP*ncaA* to heparin (an analog of the cell surface receptor heparan sulfate proteoglycan), plasma fibronectin, or cellular fibronectin (data not shown). These data indicate that NcaA promotes specific adherence to type I collagen and not to related ECM proteins or cell surface adhesion molecules.

To determine if NcaA was sufficient to confer adherence to type I collagen, we tested the binding phenotype of *E. coli* expressing *H. ducreyi* NcaA. *E. coli* TOP10 containing the *ncaA* gene in pCR2.1 (TOP10 p*ncaA*) or with pCR2.1 vector alone (TOP10 pCR2.1) were fluorescently labeled and incubated in microplate wells coated with type I collagen. Approximately 10^6 TOP10 p*ncaA* CFU bound to collagen following incubation with 8 x 10^6 CFU, whereas fewer than 100 TOP10 pCR2.1 CFU remained after incubation with 3.2 x 10^7 CFU (Fig. 3.4). TOP10 p*ncaA* did not bind wells coated with plasma fibronectin (data not shown). These data indicate that NcaA is both necessary and sufficient to elicit specific association with type I collagen.

Effect of NcaA expression on *H. ducreyi* serum resistance and adherence to keratinocytes and foreskin fibroblasts.

The Oca proteins UspA2, (2), YadA (7, 35) and Eib proteins A, C, D, E, and F (51, 52) impart resistance in their respective organisms to complement-mediated killing in the absence of opsonizing antibody, and have also been shown to mediate binding to host receptors. NcaA has significant amino acid sequence similarity to DsrA, an *H. ducreyi* outer membrane protein and Oca protein that confers resistance to killing by normal human serum (19) as well as attachment to keratinocytes (15). We therefore tested the sensitivity of 35000HP*ncaA* to normal human serum. The serum-sensitive strain *H. ducreyi* FX517 (35000HP*dsrA*) was effectively killed by 25% and 50% naïve pig serum with complement, whereas 35000HP and 35000HP*ncaA* were both resistant to complement-mediated killing (Fig. 3.5). We also determined if NcaA mediates adhesion

to keratinocytes and fibroblasts, which are the predominant cell types in skin. Both 35000HP and 35000HP*ncaA* adhered to HaCaT cells, a human keratinocyte cell line (13) and HS27 cells, a human foreskin fibroblast cell line (1634-C, American Type Culture Collection, Manassas, VA) (data not shown). From these findings we concluded that NcaA neither contributes to serum resistance nor promotes adhesion to skin fibroblasts or keratinocytes *in vitro*.

NcaA expression is necessary for virulence in the swine model of chancroid infection.

To determine if NcaA is important in skin lesion formation *in vivo* we inoculated pigs with 35000HP or 35000HP*ncaA* and assessed bacterial recovery from biopsies taken two or seven days post-inoculation (p.i.). These data were generated from 14 animals across 6 experimental iterations. The percentage of biopsies containing viable *H. ducreyi* at day 2 p.i. was not significantly different between sites inoculated with 35000HP or 35000HP*ncaA* (60.3% versus 56.4% culture-positive, respectively; Table 3.1). However there was a statistically significant difference ($P < 0.0001$) between the proportions of culture-positive biopsies taken at day 7 p.i. from sites inoculated with 35000HP (72.9%) or 35000HP*ncaA* (20.0%). Despite similar numbers of culture-positive biopsies at day 2 p.i., the average number of CFU recovered from 35000HP-inoculated sites was over 5-fold higher than the number recovered from mutant-inoculated sites (161.0 vs. 30.4 CFU per biopsy). This span increased to over 24-fold at day 7 p.i. (146.8 vs. 6.1). Differences in number of CFU recovered per biopsy were statistically significant ($P < 0.0001$) at both

days biopsied. We also inoculated pigs with the cointegrate merodiploid strain generated during construction of 35000HP*ncaA* in order to determine if the phenotype of 35000HP*ncaA* was due to a spurious attenuating mutation rather than the targeted inactivation of the *ncaA* locus. Unlike 35000HP*ncaA*, the cointegrate was recovered from pig ear lesions with similar frequency and in similar amounts to wild type 35000HP at days 2 and 7 p.i. (Table 3.1), demonstrating that this strain is not attenuated.

Human inoculation experiments.

The human challenge model allowed us to assess the importance of NcaA in establishing infection in the natural host of *H. ducreyi*. An escalating dose-response study was used to compare the virulence of 35000HP and 35000HP*ncaA*. Eleven healthy adults (seven females, four males; age range of 20 to 53; mean age $\pm$ the standard deviation, 38.6 $\pm$ 11.3) volunteered for the study. One subject (245) withdrew prior to inoculation. In the first iteration, one subject was inoculated at 3 sites with an EDD of 34 CFU of the parent and at three sites with EDDs of 23, 46, and 92 CFU of the mutant. Papules developed at 2 of 3 sites inoculated with the parent and 2 of 3 sites inoculated with the mutant, but all the papules resolved (Table 3.2). In the second iteration, 3 subjects were inoculated at 3 sites with an EDD of 85 CFU of the parent and at 3 sites with EDDs of 31, 62, and 124 CFU of the mutant. Papules developed at 4 of 9 sites inoculated with the parent strain and at 5 of 9 sites inoculated with the mutant (Table 3.2). A pustule developed at 1 parent site while all mutant sites resolved (Table 3.2). In the third

iteration, the subjects were inoculated with an EDD of 92 CFU of the parent and with EDDs of 74, 147, and 294 CFU of the mutant. Papules developed at all 9 parent and at 7 of 9 mutant sites (Table 3.2). Pustules developed at 6 of 9 parent sites, while all mutant papules resolved. In the fourth iteration, the subjects were inoculated with an EDD of 140 CFU of the parent, and EDDs of 490, 960, and 1920 CFU of the mutant. Papules developed at 8 of 9 sites inoculated with the parent and at 8 of 9 sites inoculated with the mutant (Table 3.2). A pustule developed at 1 parent site, while all mutant sites resolved (Table 3.2).

The cumulative results for the four iterations showed that papules developed at 76.7% (95% CI, 55.9%-97.4%) of sites inoculated with 35000HP and at 74.3% (95% CI, 55.3%-91.3%) of sites inoculated with 35000HP*ncaA*. Overall, pustules formed at 8/30 (26.7%; 95% CI, 6.4%-46.9%) sites inoculated with 35000HP compared to 0 of 30 (0%; 95% CI, 0%-25.9%) sites inoculated with 35000HP*ncaA* ($P = 0.005$). Thus, the mutant was impaired in its ability to form pustules when compared to the parent.

For the four parent and four mutant broth cultures used to prepare the inocula, all 192 parent colonies and all 192 mutant colonies tested were phenotypically correct (mutant, Cm^r (chloramphenicol resistant); parent, Cm^s). Of the 30 sites that were inoculated with the parent, 6 (20%) yielded at least one positive surface culture, while 0 of 18 mutant sites yielded a positive surface culture. All colonies obtained from surface cultures (n=346) and biopsy specimens (n=9) from parent sites were phenotypically correct (Cm^s). Thus, all tested colonies from the inocula, surface cultures and biopsy

specimens had the expected phenotype. We were unable to test the merodiploid strain for complementation of the 35000HP*ncaA* phenotype because inoculation of human volunteers with strains that harbor plasmids is not permitted.

Discussion

During the course of identifying targets of immune serum that conferred protection against *H. ducreyi* infection in the swine chancroid model, we found that at least one of the antibodies bound a previously uncharacterized outer membrane protein. We named this protein NcaA because our findings have shown it to be necessary for collagen association in *H. ducreyi*. The sequence of the C-terminal 79 amino acids of NcaA is similar to that of the oligomeric coiled adhesin (Oca) subfamily of autotransporter proteins. Oca proteins form oligomeric lollipop-shaped surface projections on the bacterial outer membrane consisting of N-terminal head and neck domains followed by a coiled-coil stalk and a YadA-like C-terminal domain that anchors the protein in the outer membrane (28, 48). The amino acid sequence of NcaA is consistent with this proposed structure, but its three-dimensional structure has not been determined. Oca proteins also migrate as heat-stable oligomers during SDS-PAGE. This peculiarity is thought to reflect the avid oligomerization property of these proteins on the bacterial surface (3, 19, 36, 48, 52). YadA exists as a trimer both when anchored to the bacterial outer membrane and during migration through SDS-PAGE (48). Monomeric

NcaA has a predicted molecular mass of 33 kDa but shows an apparent size of >100 kDa in SDS-PAGE (Fig. 3.2), suggesting that it also oligomerizes, though it is not known how many NcaA monomers assemble to form a functional complex.

Chancroid transmission occurs when *H. ducreyi* penetrates the keratinized skin surface layer and colonizes the dermis or epidermis of the skin. Type I collagen is the predominant structural component of skin (6) and *H. ducreyi* is found associated with type I collagen in the dermis of biopsy samples collected from human volunteers 48 hours post-inoculation (8). Other ECM attachment studies also demonstrated that *H. ducreyi* binds to immobilized type I collagen *in vitro* (1, 9). Our results indicate that specific association of *H. ducreyi* with type I collagen is mediated by NcaA. Collagen binding is a crucial factor in the pathogenesis of other organisms that inhabit skin during infection. For example, loss of YadA-mediated collagen binding is associated with loss of *Yersinia enterocolitica* virulence in a mouse model of infection (56). In addition, the *Staphylococcus aureus* collagen adhesin Cna is required for virulence in multiple animal models of infection (18, 26, 44, 46). Our finding that NcaA binds collagen is consistent with the nature of *H. ducreyi* as a dermal pathogen.

Our *in vitro* experiments showed that NcaA neither mediates adherence to cultured skin cells nor confers resistance to complement-mediated killing. We also demonstrated that NcaA is necessary and sufficient for bacterial association with type I collagen. Bacterial adherence to host tissues is generally considered to be an essential step in establishing infection (10, 43). Our studies here illustrate that adhesins mediating

association of bacteria with structural ECM proteins in contrast to adherence to host cells can also be important virulence determinants. NcaA appears to have such a role in *H. ducreyi* pathogenesis. In pigs, the *ncaA* mutant was recovered from significantly fewer lesions than wild type. In human volunteers, the *ncaA* mutant was unable to form pustules, making NcaA the seventh *H. ducreyi* protein shown to be essential for virulence in the native human host (4, 12, 21, 29, 53). Whereas these findings demonstrate that NcaA is required for infection of humans and pigs, we are conducting further studies to elucidate the collagen binding property of NcaA and to determine whether it is directly responsible for the contribution of NcaA to *H. ducreyi* pathogenesis. We are also determining if an antibody response against NcaA can confer protection against *H. ducreyi* infection.

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studies. We also thank Martha Greenwald and Beth Zwickl for enrolling the volunteers as well as the volunteers who participated in the trial.

Attributions

I performed RT-PCR studies to show that the *ncaA* inactivation in 35000*ncaA* was not exerting a negative polar effect on downstream genes, and carried out Western blot analysis confirming that strain 35000*ncaA* did not express NcaA. I also assisted with several pig experiments designed and performed by Leah Cole, and I designed and performed additional pig experiments showing complementation with the 35000*ncaA* merodiploid strain. I aggregated data from these pig experiments and performed statistical analysis. Tom Kawula performed experiments showing NcaA mediated binding of *E. coli* to collagen. Chris Elkins performed the radioimmunoprecipitation and Kristen Toffer identified D15, HhdB, and NcaA as targets of *H. ducreyi*-immune pig antibodies. Leah Cole performed the remainder of this work.

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Table 3.1. Response to experimental inoculation of *H. ducreyi* 35000HP, 35000HP*ncaA*, and 35000HP*ncaA* merodiploid in pigs.

Strain	*H. ducreyi* CFU per lesion biopsy[a]		Percent *H. ducreyi*-positive biopsies[b]	
	Day 2 p.i.	Day 7 p.i.	Day 2 p.i.	Day 7 p.i.
35000HP	161.0 (90.5-497.4)	146.8 (111.2-320.8)	60.3 (30.9-75.7)	72.9 (57.5-96.8)
35000HP*ncaA*	30.4 (19.5-82.3)[c]	6.1 (2.2-28.6)[c]	56.4 (31.6-68.7)	20.0 (17.1-61.7)[c]
35000HP*ncaA* merodiploid	280.9 (181.0-1390.2)	320.3 (148.3-1080.7)	70.0 (24.9-85.4)	75.0 (64.3-95.7)

[a] Values indicate the observed mean CFU per biopsy with 95% confidence intervals as modeled by Poisson regression and GEE adjustment to account for variability between pigs.

[b] Values indicate the observed mean proportion of biopsies harboring viable *H. ducreyi* with 95% confidence intervals as modeled by logistic regression and GEE adjustment.

[c] Significantly different (all $P < 0.0001$) from values from wild type or merodiploid lesions on same day post-inoculation.

Table 3.2. Human volunteer response to inoculation of live *H. ducreyi* strains[a]

Volunteer	Gender[b]	Days of Observation	Strain	No. of Initial Papules	Final Outcome of Initial Papule	
					No. Pustules	No. Resolved
244	F	5	35000HP	2		2
			35000HP*ncaA*	2		2
243	F	9	35000HP	3	1	2
			35000HP*ncaA*	2		2
247	M	3	35000HP	1		1
			35000HP*ncaA*	0		
248	F	5	35000HP	0		
			35000HP*ncaA*	2		2
249	M	8	35000HP	3	1	2
			35000HP*ncaA*	2		2
250	F	7	35000HP	3	3	
			35000HP*ncaA*	3		3
251	M	8	35000HP	3	2	1
			35000HP*ncaA*	3		3
242	F	6	35000HP	3		3
			35000HP*ncaA*	3		3
252	F	9	35000HP	3	1, *c*	1
			35000HP*ncaA*	3		3
253	F	5	35000HP	2		2
			35000HP*ncaA*	2		2

[a]Volunteer 244 was inoculated in iteration #1. Volunteers 243, 247 and 248 were inoculated in iteration #2. Volunteers 249, 250, and 251 were inoculated in iteration #3. Volunteers 242, 252, and 253 were inoculated in iteration #4.

[b] M, male; F, female.

[c]One site remained a papule at the time the subject achieved endpoint.

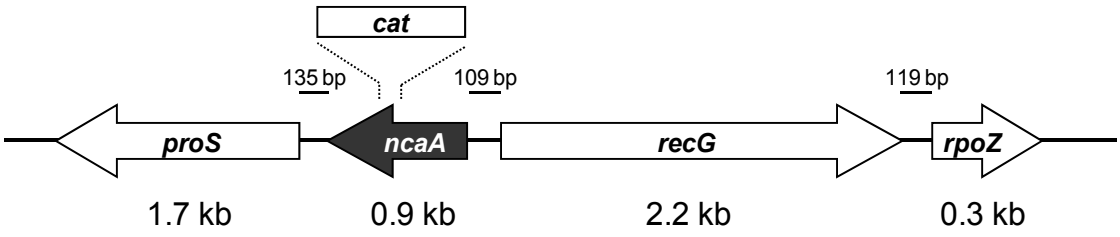

Figure 3.1. The *H. ducreyi* 35000HP *ncaA* locus and neighboring genes.
RNA transcripts of prolyl tRNA ligase (*proS*, HD1919), ATP-dependent DNA helicase (*recG*, HD1921), and RNA polymerase ω subunit (*rpoZ*, HD1923) were detected by RT-PCR and found to be produced in both 35000HP and 35000HP*ncaA* (in which the *ncaA* gene is insertionally inactivated by *cat* as shown).

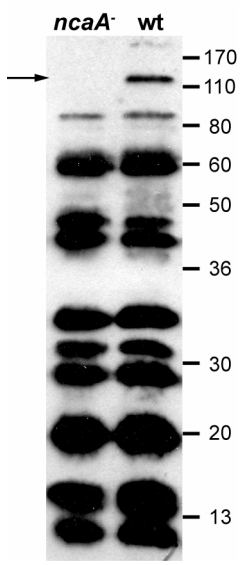

Figure 3.2. Immunoblot of *H. ducreyi* 35000HP (wt) and 35000HP*ncaA* (*ncaA*$^-$) whole cell lysates using immune pig serum.
Arrow indicates the position of an apparently multimeric form of NcaA, which migrated as a >100 kDa protein despite heating in the presence of SDS and β-mercaptoethanol. Other bands represent the constellation of *H. ducreyi* proteins recognized by polyclonal immune pig serum. Numbers indicate molecular weight standards in kilodaltons. The immunoblot film was scanned with an HP ScanJet 3970. The contrast of the scanned image was adjusted using the auto contrast command within Adobe Photoshop CS, and annotations were added with Adobe Illustrator CS.

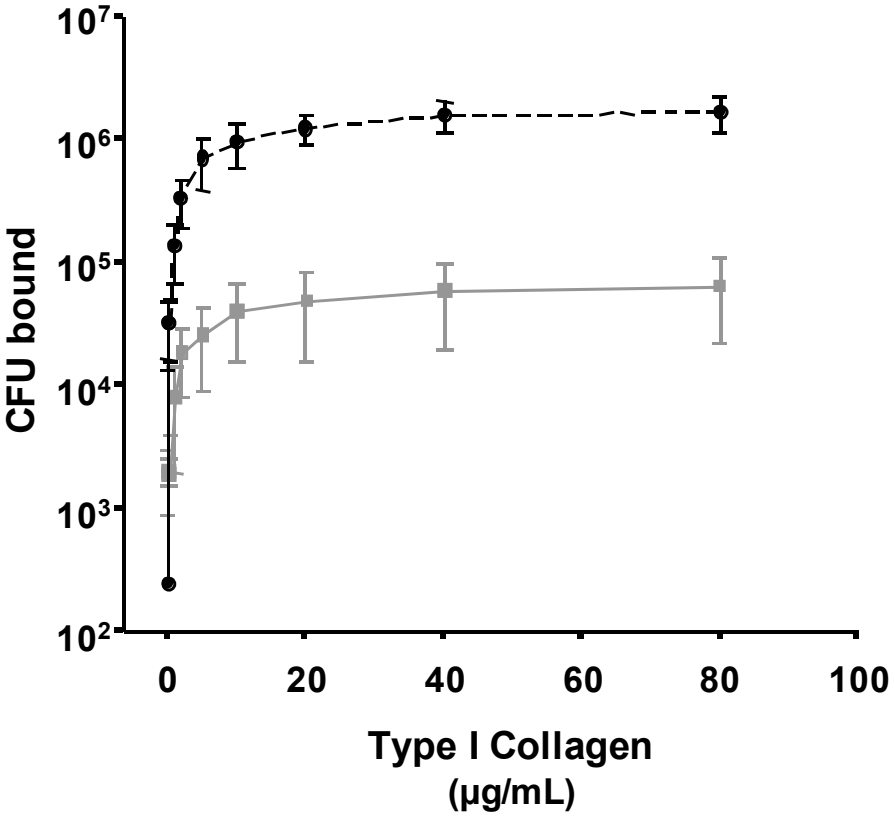

Figure 3.3. Adherence of fluorescently labeled *ncaA* mutant (grey plot) or wild type (black dashed plot) *H. ducreyi* to immobilized type I collagen.
Adherence at each concentration of collagen was tested eight times in each assay and adherence assays were performed three times for each strain. Data presented were averaged from the three assays and error bars depict ± SEM. All values except the lowest (0.002 µg/mL collagen) were significantly different (all $P < 0.05$).

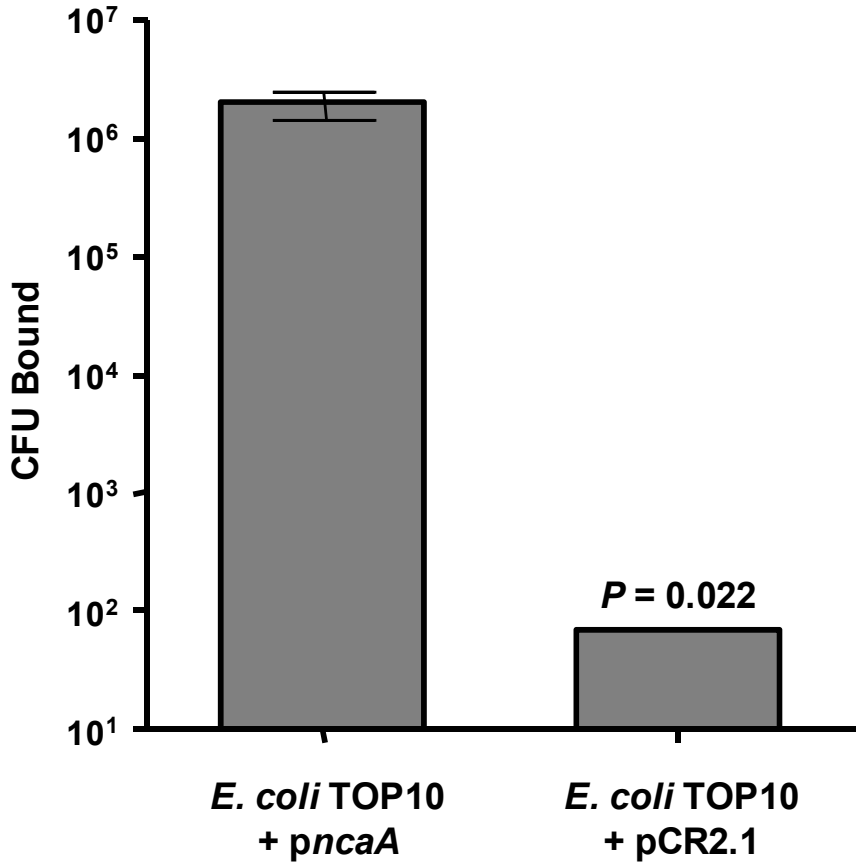

Figure 3.4. Impact of NcaA expression on *E. coli* adherence to type I collagen. Fluorescently labeled *E. coli* TOP10 containing empty vector or *ncaA* plasmids were incubated in microplate wells coated with 40 μg/ml bovine type I collagen. Data presented are mean *E. coli* CFU bound (error bars show ± SEM) following incubation with 1.6 x 10^7 and 8.0 x 10^6 vector or *ncaA* containing CFU respectively.

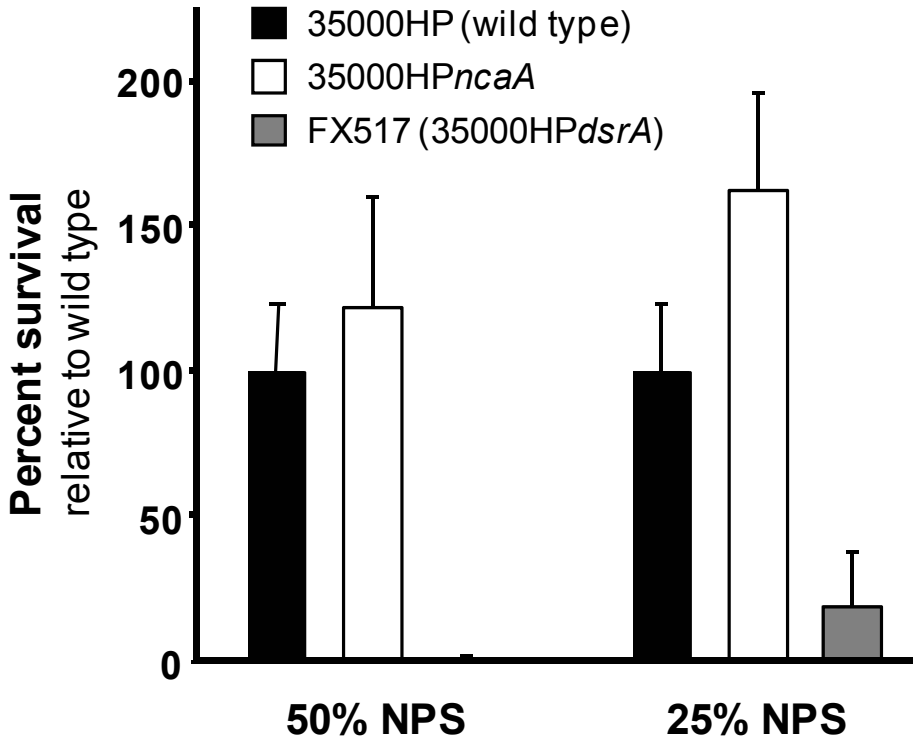

Figure 3.5. Serum sensitivity of *H. ducreyi* mutants.
Survival of wild type (black bars), *ncaA* mutant (white bars), and *dsrA* mutant (grey bars) *H. ducreyi* after incubation in 25% or 50% normal pig serum. Serum survival was assayed in triplicate in three independent experiments (error bars show ± SEM).

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CHAPTER 4

The Role of Cytolethal Distending Toxin in *Haemophilus ducreyi* Virulence

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Chancroid is a genital ulcer disease that is associated with increased risk of HIV transmission and acquisition. It is caused by the gram-negative bacterium *Haemophilus ducreyi*, and infection with *H. ducreyi* does not elicit protective immunity. *H. ducreyi* expresses cytolethal distending toxin (CDT), a soluble tripartite (CdtABC) protein complex that intoxicates and kills a range of eukaryotic cell types including some involved in host immunity. We predicted that CDT is important for *H. ducreyi* virulence and may serve to modulate host immunity. To test this hypothesis we generated *H. ducreyi* mutants unable to express the three CDT subunits (Δ*cdtABC*), tested soluble CDT activity in a cell culture system, and tested virulence in an experimental pig model of chancroid. These Δ*cdtABC* strains generated culture supernatants non-toxic to HaCaT cells *in vitro*, and were highly attenuated in pigs. However, the virulence of the Δ*cdtABC* strains within the pig model was not restored by *cis*- or *trans*-complementation with wild type *cdtABC*. Thus, these results support but do not prove the essentiality of CDT to *H.*

ducreyi virulence in pigs. In addition, repeated inoculation with *cdtABC*-null strains did not protect pigs against subsequent wild type challenge, suggesting that the absence of CDT during *H. ducreyi* infection does not lead to increased immunity to later challenge, and that CDT is not an immune modulator. We also examined the role of nuclease activity in CDT toxicity by testing CdtB amino acid variants designed to lack DNase activity. Supernatants from *H. ducreyi* strains expressing double-mutant CdtB (CdtB H160A, D199A; dmCdtB) maintained CDT toxicity against cultured cells, and recombinant purified dmCdtB digested plasmid DNA *in vitro*. The fact that amino acid-substituted CdtB variants retained activity demonstrates plasticity the in active site amino acid requirements for CdtB nuclease activity, but does not exclude or confirm nuclease activity as the primary means of CdtB-mediated toxicity.

Introduction

Haemophilus ducreyi, the causative agent of the genital ulcer disease (GUD) chancroid, is rare in developed nations but endemic in impoverished tropical regions of Africa, Asia, Latin America, and the Caribbean (37). Studies show that GUD, including chancroid (27), facilitate transmission of other sexually transmitted infections, including human immunodeficiency virus, or HIV (8, 41). Given the geographical overlap of chancroid and HIV and the established synergistic relationship between the two

pathogens (52) curtailment of chancroid is an important step in controlling the spread of HIV.

A primary concern with global management of chancroid is that infected individuals fail to develop protective immunity following infection (6, 25). This property allowed Auguste Ducrey in the late 1800s to initially characterize *H. ducreyi* by inoculating chancroid patients' forearm skin with exudate from their ulcers, and repassaging matter from the resulting lesions for as many as 15 iterations (15, 16). Humans with naturally-acquired chancroid generate *H. ducreyi*-specific antibodies that increase in titer over prolonged infection, but there has been no correlation of antibody titer and protection against reinfection (2, 14, 38). Furthermore, human *H.ducreyi*-immune sera are not effectively bactericidal against *H. ducreyi in vitro* (22). In an experimental model of chancroid, pigs repeatedly inoculated with the same *H. ducreyi* strain mounted a humoral response, though modest in titer and in efficacy upon passive transfer of immune sera to naïve animals (9, 26). Similarly, human volunteers inoculated with *H. ducreyi* remain susceptible to rechallenge with the same strain (4). Such experimental infections do not accurately represent the successive encounter of different strains as in natural infection but do create a scenario for the potential development of protective immunity to chancroid. Failure of *H. ducreyi* exposure to elicit a protective humoral response of high titer even under successive experimental challenge with homologous strains suggests that *H. ducreyi* may actively evade or modulate the host immune response. Immune evasion mediated by *H. ducreyi* could enhance its survival in

the host and thereby increase odds of transmission—an important quality for an obligate human pathogen. Extension of such immunomodulation to other bystander pathogens may also explain the increased susceptibility of chancroid-infected persons to other STI.

Among the factors mediating *H. ducreyi* virulence against eukaryotic cells is cytolethal distending toxin (CDT). This toxin is so named because CDT-intoxicated cells undergo cell cycle arrest but continue to grow, often developing a characteristic distended morphology before ultimately undergoing apoptotic cell death (12, 21). CDT is expressed by a host of pathogenic gram-negative bacterial species, including *Haemophilus*, *Actinobacillus*, *Campylobacter*, *Shigella*, *Escherichia*, and *Salmonella*. Encoded by the *cdtABC* locus, the *H. ducreyi* CdtA, CdtB, and CdtC proteins assemble to form a tripartite holotoxin complex that is lethal to a wide range of eukaryotic cells including keratinocytes, endothelial cells, and fibroblasts, which are all present within the dermal locale of the chancroid lesion (10, 11). In this manner, *H. ducreyi* CDT may contribute to lesion formation or persistence by killing the cells responsible for skin maintenance and repair (48). CDT can also intoxicate cells relevant to immune function such as lymphocytes, macrophages, and dendritic cells (44, 46, 49). An "immunosuppressive factor" discovered in the periodontal disease pathogen *Actinobacillus actinomycetemcomitans* that kills T and B lymphocytes and macrophages was later shown to be CDT (45). However, direct evidence for immune cell toxicity of *H. ducreyi* CDT within the host has not been demonstrated.

Though it is clear that all three Cdt subunits are required for full CDT toxicity, CdtB is widely acknowledged to be the primary toxigenic subunit of the CDT holotoxin (13, 23, 32, 34, 47). CdtB shares significant structural homology with bovine deoxyribonuclease (DNase) I, and recombinant *H. ducreyi* CdtB has been shown to mediate scission of double-stranded DNA *in vitro* (39). Toxicity and *in vitro* DNase activity were reduced or neutralized in recombinant *H. ducreyi* (39), *C. jejuni* (31), and *E. coli* (19) CdtB variants in which amino acids homologous to critical catalytic or metal-binding residues of DNase I activity were changed. Furthermore, introduction of recombinant CdtB alone into the cytoplasm of eukaryotic cells recapitulates cytopathology seen in holotoxin-exposed cells (31, 35, 36). Although these findings demonstrate a strong link between CdtB nuclease activity and CDT cytotoxicity, it has not been established whether CdtB-associated genotoxicity is the sole toxic determinant of CDT natively expressed by the bacterial pathogen.

Despite the obvious cytotoxic properties of CDT, studies in a human experimental model of chancroid demonstrated that CDT is not essential for infection (54). However, this study employed *H. ducreyi* 35000.303, a *cdtC* deletion mutant that expresses CdtA and CdtB, but culture supernatant from this strain lacked CDT activity (47). Though it has not been shown whether this strain secretes CdtA and CdtB in the absence of CdtC expression, CdtC is required for assembly of fully active CDT holotoxin (39, 43). The ability of 35000.303 to express CdtB brings into question the complete absence of CDT toxicity, since it is possible that CdtB is released upon lysis or uptake of *H. ducreyi* by

macrophages or dendritic cells, where the putative membrane binding or internalization functions of CdtA and CdtC may not be required. This concern is further supported by the more recent finding that a strain of *Salmonella enterica* Typhi that does not carry *cdtA* or *cdtC* genes secretes a CdtB homolog via a *sec*-dependent pathway upon engulfment of bacteria by epithelial cells (24); in this strain, CdtB alone was sufficient to intoxicate eukaryotic cells.

We propose that the most stringent test of the contribution of CDT to *H. ducreyi* pathogenesis is to assess the virulence of an *H. ducreyi* strain lacking all three CDT components. Further, we hypothesize that CDT-deficient *H. ducreyi* will be attenuated, and may evoke a stronger protective immune response in the host following repeated inoculation in the absence of CDT-mediated immune modulation. To test these hypotheses we constructed a *cdtABC*-deficient *H. ducreyi* strain and characterized its virulence in both *in vitro* assays and an *in vivo* model of *H. ducreyi* infection. In addition, we investigated whether DNase activity is the sole or primary mode of CDT-mediated cytotoxicity by testing the activity of CdtB mutants in which residues predicted to be essential for DNase activity were altered.

Materials and Methods

Growth of bacterial cultures.

Haemophilus ducreyi cultures were grown on chocolate agar (25 g/L brain heart

infusion [BD, Franklin Lakes, NJ], 10 g/L freeze-dried hemoglobin [MP Biomedicals, Solon, OH], 1% IsoVitaleX [BD], 5% fetal calf serum [Sigma-Aldrich, St. Louis, MO or Gibco-Invitrogen, Grand Island, NY], 5% newborn calf serum [Gibco-Invitrogen], and 1.5% agar) or in complete BHI broth (37 g/L brain heart infusion, 50 μg/mL hemin [Sigma-Aldrich], 1% IsoVitaleX, and 10% fetal calf serum). Antibiotics were included as indicated: 10 μg/mL chloramphenicol (Cm), 30 μg/mL kanamycin (Kn), 3 μg/mL vancomycin (Vc) [all Sigma-Aldrich]. *Escherichia coli* cultures were grown in LB broth consisting of 10 g/L tryptone, 5 g/L yeast extract [BD], and 10 g/L NaCl, or on LB agar comprised of LB broth with 15 g/L agar [USB]. Antibiotics were included as indicated: 100 μg/mL ampicillin (Ap), 35 μg/mL chloramphenicol (Cm), 40 μg/mL kanamycin (Kn). Recovery medium for *E. coli* and *H. ducreyi* electroporation (SOC) consisted of 20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.

Mutagenesis of the *H. ducreyi cdtABC* locus.

H. ducreyi Δ*cdtABC* mutant ΔCDT1 was constructed by single-step, double-crossover allelic exchange. The mutagenesis construct was a linear DNA segment consisting of a chloramphenicol resistance (Cm^R) gene placed within the flanking sequence of the wild type *cdtABC* locus. The chloramphenicol acetyltransferase (*cat*) cassette of pUNCH40 (51) was first isolated by *Bgl* II digestion. Next, a 4.2 kb region of the *H. ducreyi* 35000 genome centered on the 2.2 kb *cdtABC* locus was amplified by PCR

using primers HdOSU720F and HdOSU720R. This product was cloned into pCR2.1-TOPO [Invitrogen] to produce pRAF30. pRAF30 was digested with *Nde* I (190 bp upstream of *cdtA* start site) and *Sgr*A I (18 bp upstream of the *cdtC* stop codon) to remove the *cdtABC* locus. The *Nde* I/*Sgr*A I-digested pRAF30 backbone and the *cat* cassette insert were blunt-ended with Klenow polymerase [New England Biolabs, Ipswich, MA] and ligated to create pRAF31. The *cdt*-flanked *cat* cassette was digested from the pRAF31 backbone with *Kpn* I and *Xho* I, gel purified on 1% agarose-TAE and electroporated into *H. ducreyi* 35000 as linear DNA. Bacteria grown overnight on chocolate agar were swabbed into cBHI and used to inoculate a 60 mL of cBHI to a turbidity of 40 as measured on a Klett-Summerson photoelectric colorimeter. The culture was grown at 35°C for 2 h (Klett = 90) and harvested by centrifugation (5 min, 1000 x *g*, RT). Pellets were washed 4 times with 1 mL 10% glycerol and resuspended in 100 µL 10% glycerol and held on ice. Electroporated (2.5 kV, 25 µF, 200 Ω) bacteria were rescued in 900 µL SOC, then added to 4 mL of non-selective cBHI and grown for 2 h at 35°C. The culture was pelleted and plated on chocolate agar containing 1 µg/mL Cm; this selection yielded the Cm^R CDT-null mutant ΔCDT1. Strain ΔCDT1 was complemented by transforming with intact circular pRAF30 and plating on chocolate agar containing 1 µg/mL Cm and 10 µg/mL Kn to select both the mutant Δ*cdtABC*□*cat* allele and integration of the pRAF30 backbone. The resulting merodiploid clone was designated ΔCDT1mero.

A second *H. ducreyi* Δ*cdtABC*::*cat* mutant was created using a two-step allelic exchange procedure involving counterselection against the β-galactosidase (*lacZ*) gene as previously described (7). First, the *cdt*-flanked *cat* cassette was PCR-amplified from pRAF31 [*Pfu*Turbo, Stratagene, La Jolla, CA] using the HdOSU730F/R primer pair. Suicide vector pRSM2072 (7) was digested with *Xho* I, blunt-ended with Klenow polymerase, and ligated with *cdt*-flanked *cat* to form pRAF101. *H. ducreyi* 35000 was transformed with circular pRAF101 and plated on chocolate agar with 1 μg/mL Cm. *H. ducreyi* isolates expressing *lacZ* (carried by pRSM2072) grow slowly on media with X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside], allowing counterselection for plasmid cointegrate excision (7). pRAF101 transformants were streaked on chocolate agar containing 1 μg/mL Cm and 40 μg/mL X-gal, and relatively fast-growing, white-colored colonies were chosen yielding the Δ*cdtABC*::*cat* mutant ΔCDT2. The mutant *cdtABC*□*cat* allele of ΔCDT2 was replaced with wild type *cdtABC* using the same two-step procedure, using pRAF100K as the donor for allelic exchange. Transformants were selected on 1 μg/mL Cm and 10 μg/mL Kn and counterselected on chocolate agar with 40 μg/mL X-gal and no antibiotics. Fast-growing white colonies were patched onto fresh medium, tested for Kn- and Cm-sensitive phenotypes, and screened using the methods mentioned below. The final wild type "repaired" strain was named ΔCDT2rep.

An *H. ducreyi* 35000 mutant expressing an altered "double-mutant" (His 160 to Ala, Asp 199 to Ala) CdtB was also created by the two-step procedure outlined above. The allelic exchange construct was created by performing two stepwise QuikChange II XL

[Stratagene] reactions on pRAF100K, first with the primer pair HdCdtB(H160A)F/R, then with primer pair HdCdtB(D199A)F/R. The resulting pRAF100KBdm was used for two-step allelic exchange with wild type *cdtB* in *H. ducreyi* 35000. Following X-gal counterselection, clones were screened by amplifying the *cdtABC* locus by PCR (CdtSeqF1/R1 primers) and digesting the amplicon to test for a novel *Bsm* I site introduced by the H160A substitution. Because the double-mutant *cdtB* allele was unmarked, some isolates resolved of pRAF100KBdm carried the double-mutant allele, and some reverted to wild type. One isolate of each genotype was carried forward: strain DMB (double-mutant *cdtB*) and strain WTB (wild type *cdtB*).

The genotypes of *H. ducreyi cdt* mutants were confirmed by Southern blot analysis and DNA sequencing [UNC Genomic Analysis Facility, Chapel Hill, NC] using the Hd35kCDT+flankF/R primers (Table 2). Genotypes were further analyzed by Southern blotting. Genomic DNA isolated [MasterPure DNA purification kit, Epicentre Biotechnologies, Madison, WI] from *H. ducreyi* strains was digested with *Pst* I and resolved on 0.8% agarose-TAE, blotted to a nylon membrane [Hybond-N+, GE Healthcare, Piscataway, NJ] using capillary transfer, and UV-crosslinked [Stratalinker, Stratagene]. Digoxigenin (DIG)-labeled PCR products [PCR DIG Probe Synthesis Kit, Roche, Indianapolis, IN] corresponding to the *cdtB* gene (primers cdtB-BamHI-F/EcoRI-R, template pRAF30) or the *cat* gene (primers cdt_flank_F/R, template pRAF31) were generated and used to probe the blotted DNA fragments. Standard protocol for Southern blot hybridization was followed (5). The blot was probed with peroxidase-conjugated α-

DIG antibody, incubated with chemiluminescent substrate [Roche], and visualized by autoradiography [HyBlot CL, Denville Scientific, Metuchen, NJ].

For analysis of CDT subunit expression, whole-cell suspensions of *H. ducreyi* strains were dispersed in 1x reducing SDS-PAGE loading buffer and resolved on 12.5% tris-glycine acrylamide gel. Resolved proteins were transferred to supported nitrocellulose [Optitran BA-S 83, Whatman, Florham Park, NJ]. Membranes were blocked with 1% BSA in PBS-T (phosphate buffered saline plus 0.05% Tween-20), and probed with hybridoma supernatants containing mAb 1G8 (α-CdtA), 20B2 (α-CdtB), or 8C9 (α-CdtC) [mAbs kind gift of Eric Hansen, University of Texas-Southwestern Medical Center, Dallas, TX], followed by peroxidase-conjugated α-mouse IgG [Sigma]. Blots were developed with chemiluminescent substrate [SuperSignal Pico, Pierce, Rockford, IL] and exposed to autoradiography film.

Experimental inoculation of pigs with ΔCDT variants of *H. ducreyi*.

H. ducreyi strains were streaked onto chocolate agar for a confluent growth and incubated for 11-12 h at 35°C in 5% CO_2. The lawn was then restreaked onto 6 fresh plates and incubated for 12 h. Antibiotics were included for plasmid maintenance where indicated. Bacteria were collected and resuspended in 2 mL PBS with vortexing. The suspension was forced through a 30G ½" needle to disperse aggregates. Inoculum density was determined by serial dilution and culture on chocolate agar.

Pigs were anesthetized using 0.3 mL/kg body weight of an anesthetic-analgesic solution injected intramuscularly. The agent consisted of tiletamine HCl and zolazepam HCl 50 mg/mL each [Telazol, Fort Dodge Labs, Fort Dodge, IA], ketamine 50 mg/mL [Ketaset, Fort Dodge], and xylazine 50 mg/mL [TranquiVed, Miles Labs, Shawnee Mission, KS]. Ears were prepared by briefly but vigorously scrubbing the dorsal surface with a gauze pad moistened with water and 70% ethanol. Bacterial inoculum (10 μL) was loaded onto the tines of a Multi-Test applicator [Lincoln Diagnostics, Decatur, IL] and pressed into the cleaned ear surface. Sites were circled using Sharpie markers [Sanford Corporation, Oak Brook, IL] and photographed to record site inoculum and position. Subsets of lesions were biopsied at 2 and 7 days post-inoculation using 6 mm diameter biopsy punches [Acuderm, Ft. Lauderdale, FL] and longitudinally bisected. Half was stored in complete BHI medium and half in 4% paraformaldehyde [Polysciences, Warrington, PA] in PBS. Biopsies in BHI were minced and a defined quantity plated (usually 2 x 200 μL) on BHI + VCN for recovery. After 48h incubation, *H. ducreyi* colonies were counted to record viable bacteria remaining in the experimental lesion at 2 and 7 days following inoculation; these data were used as a measure of virulence of the tested strains.

In vitro expression of CdtB variants.

Using QuikChange II XL [Stratagene] mutagenesis as described earlier, mutations encoding CdtB H160A and D199A substitutions were introduced into pRAF30 and into

pLSCDT, a derivative of *H. ducreyi* replicative plasmid pLS88 containing the wild type *cdtABC* locus. These reactions produced the double-mutant *cdtB* constructs pRAF30dmB and pLSCDTdmB. pLSCDTdmB was further mutagenized to encode a CdtB H274A substitution (using the primer pair HdCdtB(H274A)F/R) produce the "triple-mutant" *cdtB* construct pLSCDTtmB. Wild type and double- or triple-mutant variants of *cdtB* were amplified using the primers cdtB-BamHI-F and cdtB-EcoRI-R. Wild type *cdtB* was amplified from pRAF30, whereas double- and triple- mutant *cdtB* were amplified from pLSCDTdmB and pLSCDTtmB, respectively. PCR products were digested with *Bam*H I and *Eco*R I and directionally ligated into pRSET A [Invitrogen], an N-terminal hexahistidine fusion protein expression vector. The resulting plasmids pRAF21*mat* (wild type CdtB), pRSETdmB (H160A/D199A), and pRSETtmB (H160A/D199A/H274A) were maintained in TOP10 *E. coli*, and transformed into BL21(DE3)pLysS *E. coli* for protein expression.

Recombinant CdtB expression conditions were based closely on those by Deng *et al.* (13). Overnight cultures of pRAF21*mat*, pRSETdmB, and pRSETtmB were grown in LB with 100 μg/mL Ap and 35 μg/mL Cm, diluted 1:25 into 250 mL LB with 100 μg/mL Ap and 2% glucose, and incubated with vigorous shaking at 37°C until the OD_{600} reached 0.6-0.7. Once cooled to RT, the culture was induced with 0.5 mM IPTG for 3-4 h and the bacteria were harvested by centrifugation (15 min, 8000 x *g,* 4°C) and stored at -80°C. After thawing, pellets were resuspended in 5 mL His purification buffer (HPB; 0.5 M NaCl, 5 mM imidazole in 20 mM Tris pH 8.0) with protease inhibitor [Complete Mini

protease inhibitor tablets; Roche] then freeze-thawed three times in dry ice-EtOH, then cold tap water. The suspension was sonicated intermittently on wet ice and centrifuged at 10,000 x *g* for 10 min at 4°C. Ni^{2+}-NTA agarose [Qiagen] was added to the clarified supernatant and mixed for 1 h at RT. The slurry was loaded into a 1 mL column then washed and eluted with HPB containing escalating concentrations of imidazole; CdtB typically eluted at 80 mM imidazole. Samples of each fraction were resolved by SDS-PAGE and visualized with Coomassie Brilliant Blue R-250. Fractions containing CdtB were pooled, dialyzed to remove imidazole and stored at 4°C. Concentrations of purified recombinant CdtB variants were determined by BCA protein assay [Pierce].

***In vitro* cytotoxicity testing of *H. ducreyi* culture supernatants.**

Complete BHI broth with 25 μg/mL hemin but no serum or antibiotics was inoculated with *H. ducreyi* strains from glycerol freezer stocks and incubated for 18 h at 35°C. Routinely a loopful of each culture was streaked for isolation onto chocolate agar to test for contamination; in later cultures, high turbidity alone was used as indication of contamination (saturated *H. ducreyi* cultures have relatively low turbidity). Cultures were centrifuged to remove bacteria (8000 x *g*, 15 min, 4°C) then these supernatants were ultracentrifuged to remove residual bacterial fragments (150,000 x *g*, 2 h, 4°C). After filtration through 0.2 μm filters, supernatants were aliquoted and stored at -80°C; stability has been reported at 1 month (47), but our findings suggest a two-month maximum.

HaCaT cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, β-mercaptoethanol, and 10 mM HEPES. Cells were seeded at low (20-30%) confluency in tissue culture plasticware according to the analysis method. For visual analysis, cells were cultured in clear flat-bottom 96-well microplates, or in 8-well chamber slides [Nalge Nunc, Rochester, NY]. For measurement of metabolic activity, cells were cultured in opaque white 96-well microplates [Corning, Acton, MA]. Cells were incubated overnight to allow recovery from passage and adherence.

An equal volume of supernatant was added to the tissue culture medium contained in each well to a total of 50% concentration. For titration experiments, the supernatant was diluted in complete BHI and then added as above. Each well was then washed twice with PBS, replenished with fresh culture medium, and incubated for 2 to 4 hours at 37°C in 5% CO_2. Cells were observed or assayed at 24-48 hours post-intoxication for CDT-associated cytopathology. Controls included complete BHI, and untreated cells.

Visual analysis of CDT intoxication consisted of qualitative observation of cell morphology; CDT-intoxicated cells appear distinctly enlarged relative to untreated cells. For quantitative analysis of intoxication, the fluorescent metabolic indicator dye AlamarBlue [Biosource-Invitrogen, Carlsbad, CA] was added at 10% concentration to each well and incubated for 2-6 h. Fluorescence from each well at 590 nm was measured during excitation at 530 nm using a BioTek Synergy HT [Winooski, VT] or Molecular

Devices SpectraMAX m2 [Sunnyvale, CA] microplate fluorimeter, with excitation and detection at the top of the wells.

Assessing nuclease activity of recombinant CdtB proteins.

Defined quantities of purified recombinant wild type CdtB, dmCdtB, and tmCdtB were added to a reaction mixture consisting of 5 mM $MgCl_2$, 0.4 μg/mL bovine serum albumin, 75 mM HEPES, and 0.5 μg of pUC19 [Invitrogen] plasmid DNA substrate. Two control reactions were run in parallel: one including buffer and DNA, but no protein, and one including recombinant purified β-galactosidase (LacZ) purified in parallel with CdtB variants to insure exclusion of *E. coli* nuclease activity during nickel-affinity purification. Reactions were incubated at 37°C for 4 hours and were resolved by electrophoresis through 1% TAE-buffered agarose. DNA species were stained with ethidium bromide and visualized and photographed under UV transillumination. Nuclease activity was qualitatively measured by the conversion of fast-migrating supercoiled plasmid to slow-migrating nicked or relaxed form or intermediate-migrating linear form.

Results

Construction and validation of *H. ducreyi* CDT deletion mutants.

To determine the influence of CDT on *H. ducreyi* virulence, we constructed mutants unable to express the three CDT subunits by targeted allelic exchange. The *cdtABC* locus of *H. ducreyi* wild type strain 35000 was exchanged in one double-crossover event with a chloramphenicol acetyltransferase (*cat*) gene to produce strain ΔCDT1. The mutation in ΔCDT1 was complemented *in cis* by chromosomal integration of a plasmid-borne copy of *cdtABC* to produce ΔCDT1mero. A second *cdtABC*-deletion mutant, ΔCDT2, was created by integration and resolution of the Δ*cdtABC*□*cat* allelic exchange construct pRAF101 (7). The *cdtABC* locus of strain ΔCDT2 was then restored to wild type allele using the same two-step procedure to produce the "repaired" strain ΔCDT2rep.

H. ducreyi mutant strains ΔCDT1 and ΔCDT1mero were analyzed by Southern blot to rule out multiple integration of the Δ*cdtABC*::*cat* allele or other aberrant rearrangement. A DIG-*cdtB* probe hybridized with a 4.7 kb fragment of *Pvu* I-digested 35000 gDNA and a 6.8 kb fragment of ΔCDT1mero gDNA (Fig. 4.1), consistent with integration of the *cdtABC* copy downstream of the *cat* cassette; no hybridization to ΔCDT1 gDNA was observed. A DIG-*cat* probe recognized 3.5 kb bands in both ΔCDT1 and ΔCDT1mero gDNA but did not hybridize to 35000 gDNA. These results were consistent with a single targeted allelic exchange at the *cdtABC* locus in strain ΔCDT1, as well as successful integration of pRAF30 (carrying wild type *cdtABC*) downstream of the *cat* cassette in ΔCDT1mero. Southern blot analysis of strains ΔCDT2 and ΔCDT2rep

showed that ΔCDT2 yielded identical results to ΔCDT1, and ΔCDT2rep produced results identical to wild type strain 35000 (data not shown).

We also tested CDT expression phenotypes of both ΔCDT1 and ΔCDT2 mutants and their corresponding complemented strains by probing a Western blot of whole cell lysates with 3 mAbs directed against each CDT subunit (Fig. 4.2). As expected, ΔCDT1 and ΔCDT2 lacked detectable CdtA, CdtB, or CdtC, while ΔCDT1mero and ΔCDT2rep express CDT subunits at levels similar to those of wild type *H. ducreyi*. These results confirmed that expression of the three CDT subunits was ablated in the ΔCDT strains and restored in the analogous complemented strains.

Testing virulence of *H. ducreyi* CDT deletion mutants in pigs.

CDT intoxicates a broad range of eukaryotic cells, including vascular endothelial cells, skin fibroblasts, and keratinocytes. As a pathogen that infects skin, *H. ducreyi* strains secreting CDT will likely intoxicate these cell types proximal to the chancroid lesion, thereby contributing to local pathology. Therefore, we hypothesized that CDT is important to *H. ducreyi* virulence and that CDT-deficient strains will accordingly be attenuated in models of chancroid. To test this hypothesis we inoculated pigs either with wild type *H. ducreyi* or with mutants unable to express CDT. We compared the survival of CDT-deficient strains to their wild type parent in the swine chancroid model to determine whether CDT expression is essential for *H. ducreyi* infection.

We inoculated pigs with wild type, CDT-deficient, or complemented *H. ducreyi* strains twice, separated by a two-week interval. This setup allowed us two opportunities to assess survival of these mutants in the pig, and provided prime and boost inoculations for comparing elicitation of protective immunity by these strains (described later). Virulence of each strain was determined by counting the number of viable *H. ducreyi* in lesion biopsies 2 and 7 days post-inoculation. Following inoculation, significantly fewer CFU were recovered from ΔCDT1 lesion biopsies than from 35000 lesions (Table 3), demonstrating that strains unable to express CDT were impaired for virulence. However, neither *cis*-complementation (ΔCDT1mero) nor *trans*-complementation of the *cdtABC* mutant (ΔCDT1 pLSCDT) restored virulence. Theorizing that strain ΔCDT1 may have developed a cryptic second-site mutation that was causing or augmenting its attenuated phenotype, we constructed a second CDT mutant, ΔCDT2. Pigs were inoculated with 35000, ΔCDT2, ΔCDT2 complemented with pLSCDT, or the *cdtABC*-repaired ΔCDT2rep as with the ΔCDT1 strains previously. Again, though significantly fewer CFU were recovered from ΔCDT2 lesions than from 35000 lesions, complemented strains ΔCDT2 pLSCDT (first inoculation) and ΔCDT2rep (second inoculation) were also attenuated (Table 4).

Both ΔCDT1 and ΔCDT2 were independently generated but neither could be complemented, suggesting that deletion of *cdtABC* selects for secondary attenuating mutations that reconstitution of *cdtABC* cannot recompense. Though CDT may be an essential factor in *H. ducreyi* infection, lack of complementation prevented confirmation

that the virulence phenotypes of ΔCDT1 and ΔCDT2 were solely due to the absence of CDT.

Beyond its potential role in contributing to chancroid-associated skin tissue pathology, CDT can intoxicate cells of the host immune system and thus has been implicated as an immunomodulatory agent. In this manner, CDT may have additional effects beyond the locality of the chancroid lesion. Repeated inoculation with *H. ducreyi* strain 35000 in the swine model has been shown to provoke only a modest humoral immune response (9). The reason for the low magnitude of the anti-*H. ducreyi* immune response is not currently known. We asserted that CDT may interfere with development of host immunity during *H. ducreyi* infection. A repeated inoculation scheme was devised to test this idea; two weeks after the second inoculation of the aforementioned pigs, we challenged all animals with wild type *H. ducreyi* 35000. We predicted that if CDT has a negative impact on development of host immunity to *H. ducreyi*, then pigs previously inoculated with CDT-deficient strains should be less susceptible to wild type (CDT^+) challenge than pigs previously inoculated only with wild type 35000. The number of *H. ducreyi* CFU recovered from each lesion biopsy was not significantly different, regardless of the inoculation history of the pig (Table 5). Thus, infection with strains lacking CDT did not promote development of more effective anti-*H. ducreyi* immunity.

Toxicity of *H. ducreyi* CDT mutant culture supernatants *in vitro*.

To evaluate whether CDT deletion mutants produce soluble CDT activity, supernatants from saturated broth cultures of each mutant strain were applied to cultured HaCaT cells. We evaluated the treated cells for visible signs of CDT intoxication (distension) and for loss of metabolic activity using the fluorescent indicator AlamarBlue. Supernatants derived from *cdtABC* deletion strains ΔCDT1 and 2 did not cause cellular distension of HaCaT cells even at the maximum concentration of 50 percent (Fig. 4.3). CDT-complemented strains (ΔCDT1mero, ΔCDT2rep) were as efficient as wild type in promoting distension. Distension was concordantly accompanied by loss of metabolic function (Fig. 4.4). Supernatant from ΔCDT2 pLSCDT was approximately 100-fold more toxic than supernatant from wild type bacteria in the metabolic assay. Cells exposed to ΔCDT2 pLSCDT supernatant did not appear distended, but their nuclei were markedly misshapen, and few cells remained attached to the substrate (Fig. 4.3). It is possible that ΔCDT2 carrying the multi-copy pLSCDT produced and secreted more CDT into the culture supernatant than wild type or *cis*-complemented strains, causing more rapid cell death and precluding growth-dependent distended morphology. Cells treated with supernatants from Δ*cdtABC*::*cat* strains did not show an appreciable loss of viability compared to medium-only controls. These results demonstrate a direct association of cellular distension and death with supernatants derived from strains expressing CDT, and a relative lack of cytotoxicity in ΔCDT culture supernatants.

Because ablation of the entire *cdtABC* locus in *H. ducreyi* appears to select cryptic compensatory mutations resulting in attenuation, we created an *H. ducreyi* strain that expresses CDT, but whose CdtB subunit contains minimal amino acid substitutions abolishing deoxyribonuclease activity. The altered "double-mutant" CdtB (dmCdtB) contains alanine substitutions for histidine 160 and aspartate 199 (H160A, D199A). These residues were chosen based on previous work showing that a similar variant of *H. ducreyi* CdtB (H160Q, D199S) was devoid of *in vitro* nuclease activity (39). We created two *H. ducreyi* mutants: DMB, which expresses the dmCdtB (H160A, D199A) in the context of wild type CdtA and CdtB; and WTB, a sibling of DMB in which the wild type version of the *cdtB* gene was recreated during cointegrate resolution.

Supernatants from *H. ducreyi* DMB and WTB were as toxigenic as wild type in both distension and metabolic assays. This result suggests that the amino acids in CdtB predicted to map to critical DNase I active site residues was not essential for CDT toxicity in this assay system, or that alanine substitution in these locations was not sufficient to destroy nuclease activity.

In vitro nuclease activity of purified recombinant *H. ducreyi* CdtB variants.

Because strains encoding double-mutant CdtB produced active CDT in culture supernatants, either CdtB nuclease activity is dispensable to CDT toxicity, or the amino acid substitutions in our version of dmCdtB did not eliminate nuclease activity. To distinguish between these possibilities, we incubated purified wild type or amino-acid-

substituted variants of CdtB with supercoiled plasmid to assess nuclease activity *in vitro*. We expressed hexahistidine-tagged versions of CdtB, dmCdtB, and triple-mutant CdtB (tmCdtB; H160A, D199A, H274A) in *E. coli*, then purified the proteins under non-denaturing conditions. We incubated these recombinant proteins with supercoiled plasmid DNA. Wild type CdtB and dmCdtB both displayed nuclease activity as indicated by the conversion of supercoiled plasmid to relaxed or linear form (Fig. 4.5). Furthermore, this activity was titratable (data not shown), and was not present in a preparation of hexahistidine-tagged LacZ purified in parallel from the same *E. coli* expression strain and vector as the CdtB variants. Recombinant tmCdtB did not relax or linearize plasmid DNA (Fig. 4.5). These results show that the two CdtB putative active site residues homologous to critical residues in bovine DNase I were not required for CdtB nuclease activity in *H. ducreyi*. The lack of nuclease activity in tmCdtB may indicate that H274 is particularly sensitive to substitution or that the third substitution exceeded the threshold for substitution while maintaining activity. Though these findings do not support or refute CdtB nuclease activity as the primary determinant of CDT toxicity, retention of nuclease activity following alteration of amino acids H160 and D199 is a novel finding that calls for reassessment of the functional significance of apparent CdtB-DNase I homology.

Discussion

Cytolethal distending toxin has attracted a recent flurry of attention from researchers as a means by which pathogenic bacteria might modulate host cell activity by influencing the eukaryotic cell cycle. CDT homologs have been found in a wide variety of gram-negative bacteria (46) including multiple pathogenic species. Extracts of *Shigella dysenteriae* were shown to cause diarrhea and colonic epithelial damage in a CDT- and dose-dependent manner in a suckling mouse model (40). *E. coli* O157 isolates from both humans and cattle express CDT (17, 33). CDT produced by Shiga toxin-producing *Escherichia coli* (STEC) may contribute to microvascular epithelial pathology during the development of hemolytic uremic syndrome (29). In a recent study in Bahrain (3), over 80 percent of *Campylobacter jejuni* isolates from diarrhea victims carried the *cdtB* gene. In addition, CDT is expressed by about one-third of *Actinobacillus actinomycetemcomitans* (*Aa*) isolates from human patients with periodontal disease (50). Furthermore, supernatants from CDT-positive but not CDT-negative *Aa* isolates arrest growth of periodontal ligament cells and gingival fibroblasts *in vitro*; these cell types are both essential for healthy gum and tooth maintenance. Although direct and incontrovertible association between CDT expression and clinical pathology has yet to be demonstrated in any pathogen, CDT clearly is a virulence factor with the potential to affect clinical manifestation of *H. ducreyi* infection.

Multiple studies have shown that the *H. ducreyi cdtABC* locus is present and actively expressed in 82 to 89% of *H. ducreyi* clinical isolates (1, 30, 42). It is not known whether naturally CDT-negative strains more efficiently elicit protective immunity upon successive exposure, but their reduced prevalence compared to CDT-expressing isolates hints at a selective disadvantage. Svensson *et al.* showed that recombinant *H. ducreyi* CDT expressed in *E. coli* inhibits the growth of human vascular endothelial cells *in vitro* (48), and propose that this inhibition slows angiogenesis and may explain the protracted healing process observed with chancroid ulcers. Preliminary evidence from the swine chancroid model supports this hypothesis; biopsy excision sites of Δ*cdtABC* strains appear to heal markedly faster than those of CDT-expressing strains (R. A. Fulcher, unpublished data). Delayed healing of chancroid lesions could increase the odds of transmission of *H. ducreyi* to other hosts, with the unfortunate corollary of increasing the chance of superinfection with other STI pathogens.

The main objective of the studies described here was to determine whether CDT expression affects *H. ducreyi* virulence. We addressed this question by comparing the wild type strain 35000 with a patently CDT null strain, as opposed to the *cdtC* mutant tested in the human challenge study that led to the conclusion that CDT is nonessential for *H. ducreyi* virulence (54). We found that two independently-generated *H. ducreyi* strains lacking the entire *cdtABC* locus were markedly attenuated in the pig model. However, virulence could not be restored in either of these mutants by *cis*- or *trans*-

complementation, suggesting that deletion of the entire *cdtABC* locus selected for second-site attenuating mutation(s) that have not been identified.

Having demonstrated that virulence attenuation associated with the Δ*cdtABC* allele cannot be complemented, we adopted a different strategy for eliminating CDT activity. Because the nuclease activity of CdtB is thought to play a large (or perhaps singular) role in CDT toxicity, we constructed a *H. ducreyi* mutant intended to express a nuclease-negative CdtB variant with amino acids histidine 160 and aspartate 174 exchanged for alanine. This mutagenesis strategy was also chosen because it preserved the majority of the *cdtABC* locus, ostensibly generating less selective pressure for compensatory mutation. Testing the double mutant CdtB strain would also allow us to determine whether the nuclease activity of CdtB is essential for *H. ducreyi* virulence. Our findings showed, however, that culture supernatant from the double-mutant CdtB strain DMB intoxicated cultured cells similarly to wild type supernatant. Although this result rendered DMB unsuitable as a CDT-negative strain for testing in the pig model, it brought into question both the essential nature of the two catalytic amino acids as well as the overall importance of nuclease activity in CDT toxicity.

Structural homology between *H. ducreyi* CdtB and bovine DNase I active sites shows strong superimposition of amino acid functional groups critical for DNase I activity. *H. ducreyi* CdtB residues His 160 and Asp 199 map to the same residues in bovine DNase I responsible for Mg^{2+} coordination and catalysis (20, 28). *H. ducreyi* CdtB amino acids H160 and D199 have been described as essential for *in vitro* DNA scission

(39), as have the cognate residues in *E. coli* and *C. jejuni* CdtB homologs (19, 31). Our studies, however, showed that *H. ducreyi* dmCdtB expressed in and natively purified from *E. coli* was insensitive to alanine substitution at the H160 and D199 locations; other studies showing loss of nuclease activity have substituted these locations with glutamine and serine, respectively, (31, 39). Few examples of native CdtB purification in any bacterial species can be found in the literature, and preparation methods have varied widely to include denaturation and refolding (31, 32, 36), polymyxin B extraction (18, 19), and using supernatants from *E. coli* strains expressing CdtB variants from plasmids (18, 19). Because alanine substitution leaves a relatively minimal functional group compared to the H160Q and D199S substitutions, the fact that our dmCdtB variant has intact nuclease activity is confounding. Addition of the H274A substitution appears to delete nuclease activity, but we do not know if this effect is dependent upon H160A D199A. It also appears that single-copy expression of dmCdtB in *H. ducreyi* produces soluble CDT activity, but multi-copy expression from pLSCDTdmB in the ΔCDT2 background does not (Fig. 4.4). This may indicate that the alanine substitutions reduce the stability of dmCdtB, making it less amenable to overexpression. Construction of an *H. ducreyi* mutant expressing triple-mutant CdtB is under way; assessment of soluble CDT activity from this strain may help to explore the link between CdtB nuclease activity and CDT toxicity.

Overall, our results are not incongruous with the idea that CDT may be required for virulence in the experimental pig model of *H. ducreyi* infection; indeed, all CDT-

deficient strains we tested were highly attenuated. Without complementation analysis, however, it is impossible to determine whether attenuation of the CDT mutants was solely due to the absence of CDT. The presumed compensatory mutations in CDT null strains ΔCDT1 and 2 that were not complemented by *cdtABC in cis* or *in trans* remain uncharacterized. Nevertheless, attenuation of these strains in the pig model is striking, so the target of this alleged mutation may be an important *H. ducreyi* virulence determinant. Our next goal is to compare global gene expression between 35000 and these mutants using an *H. ducreyi* genome-wide microarray. Ideally the gene or genes identified in this screen will further enlighten our understanding of the mechanism of CDT toxicity and its role in chancroid pathogenesis.

Attributions

I performed all of the work in this chapter with the exception of the following instances. Sharon Taft-Benz optimized conditions for *in vitro* CdtB-mediated plasmid scission assays and performed many iterations of the assay to confirm nuclease activity of recombinant CdtB variants. Under my direction Ryan Heiniger produced a parental *cdtABC* clone (pLSCDT) for *cis*-complementation of *H. ducreyi* ΔCDT1 and verified the CDT expression phenotype of ΔCDT1 pLSCDT. Multiple members of the Kawula Lab assisted with all pig inoculations, recovery of lesion biopsies, and associated sample processing.

Table 4.1. Bacterial strains and plasmids.

Name	Genotype/phenotype	Source [a]
H. ducreyi strains		
35000	wild type	S. Spinola
ΔCDT1	35000 Δ*cdtABC*::*cat* [Cm^R]	
ΔCDT1mero	ΔCDT1 *cdtABC*$^+$ merodiploid [Cm^R, Kn^R]	
ΔCDT2	second generation 35000 Δ*cdtABC*::*cat* [Cm^R]	
ΔCDT2rep	ΔCDT2 *cdtABC*$^+$ wild type locus repaired [Cm^S, Kn^S]	
WTB	wt *cdtB* revertant from construction of 2.2	
DMB	35000 *cdtB*(H160A D199A)	
E. coli strains		
TOP10	Cloning strain; recipient for TOPO cloning	Invitrogen
BL21Star(DE3)pLysS	Expression strain for pRSET plasmids [Cm^R]	Invitrogen
Plasmids		
pCR2.1-TOPO	PCR cloning vector [Ap^R, Kn^R]	Invitrogen
pLS88	*H. ducreyi* cloning vector [Kn^R]	(53)
pRSM2072	Suicide vector for *H. ducreyi* allelic exchange; *lacZ* [Ap^R]	(7)
pRSET A	*E. coli* N-terminal 6-His expression vector [Ap^R]	Invitrogen
pLSCDT	*cdtABC* in pLS88 [Ap^R]	R. Heiniger
pLSCDTdmB	*cdtAB*(H160A D199A)*C* in pLS88 [Ap^R]	
pRAF21*mat*	wild type mature *cdtB* in pRSET A [Ap^R]	
pRAF30	4.2-kb insert with *H.d. cdtABC* in pCR2.1-TOPO [Ap^R, Kn^R]	
pRAF30dmB	pRAF30 *cdtB*(H160A D199A)[Ap^R, Kn^R]	
pRAF30tmB	pRAF30 *cdtB*(H160A D199A H274A) [Ap^R, Kn^R]	
pRAF31	pRAF30 Δ*cdtABC*::*cat* [Ap^R, Kn^R, Cm^R]	
pRAF100	4.2-kb insert with *H.d. cdtABC* in pRSM2072 [Ap^R]	
pRAF100K	pRAF100 with *aph* gene from pLS88 in *Ahd* I site [Ap^R, Kn^R]	
pRAF100KdmB	pRAF100 *cdtB*(H160A D199A) [Ap^R, Kn^R]	
pRAF101	4.2-kb Δ*cdtABC*::*cat* from pRAF31 in pRSM2072 [Ap^R, Cm^R]	
pRSETdmB	mature *cdtB* (H160A D199A) in pRSET A [Ap^R]	
pRSETtmB	mature *cdtB* (H160A D199A H274A) in pRSET A [Ap^R]	
pRSETLacZ	*lacZ* in pRSET A [Ap^R]	R. Bourret

[a] All unattributed strains and constructs were created by the author during the course of this work.

Table 4.2. Oligonucleotides used in cloning, sequencing, and mutagenesis.

Name	Sequence (5' to 3')
cdt_flank_F	CATTCAGAAAAGATATTGAACAGG
cdt_flank_R	TTTCTTCGCCCCACTAAGG
cdtB-BamHI-F [a]	CCGGATCCATGCAATGGGTAAAGC
cdtB-EcoRI-R	GCGAATTCATTAGCGATCACGAACAAAAC
CdtSeqF1	AATAGAAATCATGGCAGTTACTTGATAC
CdtSeqR1	GTTATGATTTCCCATCATTTCTAATTTG
HdOSU730F	GGTATTATTAATCTGGTATTAGAGGATACG
HdOSU730R	GGATTTGATCTTCTACAATTTACGTTCC
HdCdtB(H160A)F [b]	GGTACTGATGTATTTTTTACAGTGGCAGCTTTGGCCACAGG
HdCdtB(H160A)R	CCTGTGGCCAAAGCTGCCACTGTAAAAAATACATCAGTACC
HdCdtB(D199A)F	GGATGGTTGTTGGTGCATTCAATCGTGCGCC
HdCdtB(D199A)R	GGCGCACGATTGAATGCACCAACAACCATCC
HdCdtB(H274A)F	CGCTCACAAATTACATCCGATGCATTTCCTGTTAGTTTTGT TCGTG
HdCdtB(H274A)R	CACGAACAAAACTAACAGGAAATGCATCGGATGTAATTTG TGAGCG
Hd35kCDT+flank1F	CGTCGGTTGTACCCAAAATCG
Hd35kCDT+flank2F	GGTGCGGTTGTCATTAAAAGTGTG
Hd35kCDT+flank3F	GCCACAGGCGGTTCTGATG
Hd35kCDT+flank4F	ACGTTAGTTCAATTTAAAGTGGTTGG
Hd35kCDT+flank5F	GTATCTCAATACGATCGATAAAGTACG
Hd35kCDT+flank1R	AATTGATGACACTAGTGACGGTG
Hd35kCDT+flank2R	TGAAGATTTAGGTGCTAAATCAGATTG
Hd35kCDT+flank3R	AAACCACACTTAACTGCTTTACCC
Hd35kCDT+flank4R	ATGCAACTTCCAGATTAGCCG
Hd35kCDT+flank5R	GCCCGTATTAGTTGGAATAAGGT
Hd35kCDT+flank6R	CGAGTCATTTGAGAAACAAGCTT
pLS88KanF	TTGGCGACCTTGCATTGCAC
pLS88KanR	CGGCGTCCAGGGAGATGTCC

[a] Underlined nucleotides indicate a novel restriction site introduced for cloning.

[b] Boxed nucleotides indicate mutagenesis site.

Table 4.3: Comparative response of pigs experimentally inoculated with *H. ducreyi* wild type and ΔCDT1 derivative strains.

Strain	First inoculation (day 0)			Second inoculation (day 14)		
	n (sites / pigs)	Day 2	Day 7	*n* (sites / pigs)	Day 14 + 2	Day 14 + 7
35000	40 / 7	564.3 (304.8-1044.6)	156.0 (60.2-403.7)	44 / 7	209.5 (76.8-571.5)	217.1 (106.3-443.4)
ΔCDT1	64 / 11	46.5 (12.3-175.8)*	1.5 (0.57-4.1)*	72 / 10	2.8 (0.87-8.7)*	22.5 (6.2-81.7)*
ΔCDT1mero	20 / 4	9.9 (4.9-20.2)*	0.050 (0.0081-0.31)*	36 / 6	58.5 (10.9-312.4)	1.7 (0.29-9.8)*
ΔCDT1 pLSCDT	12 / 2	0.083 (0.021-0.33)*	0.0* [a]	- [b]		

Values indicate mean *H.d.* CFU recovered per lesion biopsy (95% CI) on the day post-inoculation indicated as modeled by Poisson regression and GEE adjustment.
* Significantly different ($P < 0.005$) from values from 35000 (wild type) on same day post-inoculation.
[a] All replicate values were zero; absence of variance prevents calculation of 95% CI.
[b] Pigs first inoculated with ΔCDT1 pLSCDT were inoculated with ΔCDT1mero in second round and analyzed with the ΔCDT1mero group.

Table 4.4: Comparative response of pigs experimentally inoculated with *H. ducreyi* wild type and ΔCDT2 derivative strains.

Strain	First inoculation (day 0)			Second inoculation (day 14)		
	n (sites / pigs)	Day 2	Day 7	*n* (sites / pigs)	Day 14 + 2	Day14 + 7
35000	12 / 2	115.8 (29.9-448.3)	230.8 (58.2-915.3)	12 / 2	36.7 (9.22-145.7)	35.1 (8.83-139.4)
ΔCDT2	12 / 2	0.0 *[a]	0.0 *[a]	12 / 2	32.9 (8.29-130.7)	0.0 *[a]
ΔCDT2 pLSCDT	12 / 2	1.92 (0.54-7.9)*	0.0 *[a]	-	-	-
ΔCDT2rep	-	-	-	12 / 2 [b]	0.0 *[a]	0.0 *[a]

Values indicate mean *H.d.* CFU recovered per lesion biopsy (95% CI) on day indicated post-inoculation as modeled by Poisson regression and GEE adjustment.
* Significantly different ($P < 0.0001$) from values from 35000 (wild type) on same day post-inoculation.
[a] All replicate values were zero; absence of variance prevents calculation of 95% CI.
[b] Pigs first inoculated with ΔCDT2 pLSCDT were inoculated instead with ΔCDT2rep on day 14.

Table 4.5. Response of pigs challenged with *H. ducreyi* 35000 after previous inoculations with *H. ducreyi* ΔCDT1 derivative strains.

Inoculation history [a]	*n* (sites / pigs)	Day 28 + 2	Day 28 + 7
2 x 35000	38 / 6	275.2 (87.22-868.4)	153.8 (42.23-560.5)
2 x ΔCDT1	72 / 11	305.8 (165.8-564.2)	223.0 (96.96-512.8)
2 x ΔCDT1mero	24 / 4	139.2 (101.0-191.7)	111.0 (53.62-230.0)
1 x ΔCDT1 pLSCDT, 1 x ΔCDT1mero	12 / 2	228.5 (164.5-317.6)	177.4 (50.21-626.9)

Values indicate *H. ducreyi* 35000 CFU recovered per biopsy (95% CI) as modeled by Poisson regression and GEE adjustment.

[a] Pigs were previously inoculated at days 0 and 14 with indicated strains, then challenged with 35000 on day 28.

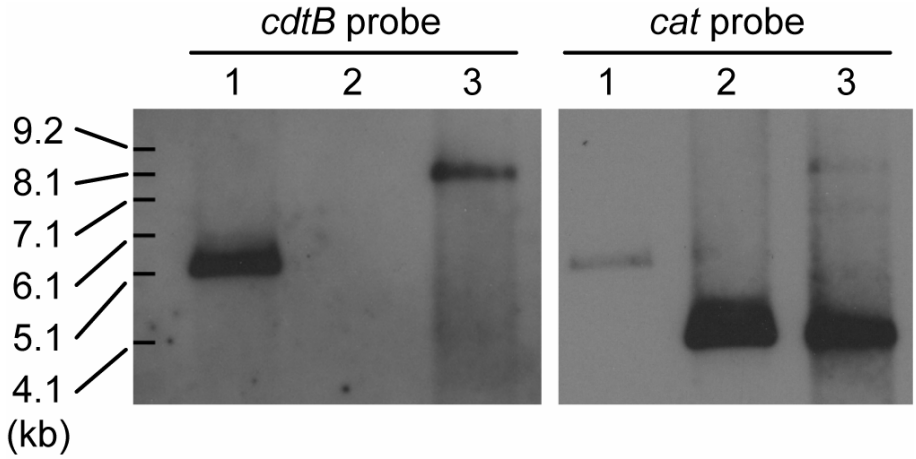

Figure 4.1. Confirmation of CDT genotypes by Southern blot.
Pvu I-digested genomic DNA from strains 35000 (lane 1), ΔCDT1 (lane 2), and ΔCDT1mero (lane 3) were probed with DIG-labeled *cdtB* (left panel). The same membrane was stripped and reprobed with DIG-*cat* (right panel). Residual *cdtB* signal remains in the *cat* blot due to incomplete probe removal and was used to orient the two images. Marker locations were translated from measurements of the original agarose gel pre-transfer, and are approximate. Image was assembled in Adobe Photoshop CS, maintaining identical scale between the scans of the two autoradiographs.

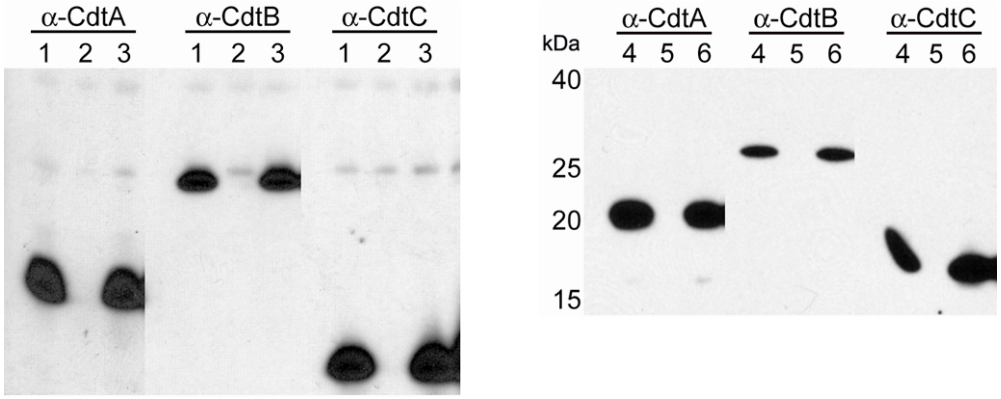

Figure 4.2. Confirmation of *H. ducreyi* CDT-null mutant expression phenotypes. Standardized amounts of *H. ducreyi* 35000 (lanes marked 1, 4), ΔCDT1 (lanes 2), ΔCDT1mero (lanes 3), ΔCDT2 (lanes 5), and ΔCDT2rep (lanes 6) whole-cell lysate were resolved on SDS-PAGE and transferred to nitrocellulose, and probed with α-CdtA (1G8), α-CdtB (20B2), or α-CdtC (8C9) monoclonal antibodies. Strains ΔCDT1 and ΔCDT2 have the Δ*cdtABC*::*cat* genotype, ΔCDT1mero is a Δ*cdtABC*::*cat cdtABC*$^{+}$ merodiploid, and ΔCDT2rep is a version of ΔCDT2 restored to wild type genotype. Each of these two panels of blots was derived from a single SDS-PAGE gel, but each image was abridged using Adobe Photoshop CS. The relative positions of the different Cdt species in each panel were preserved.

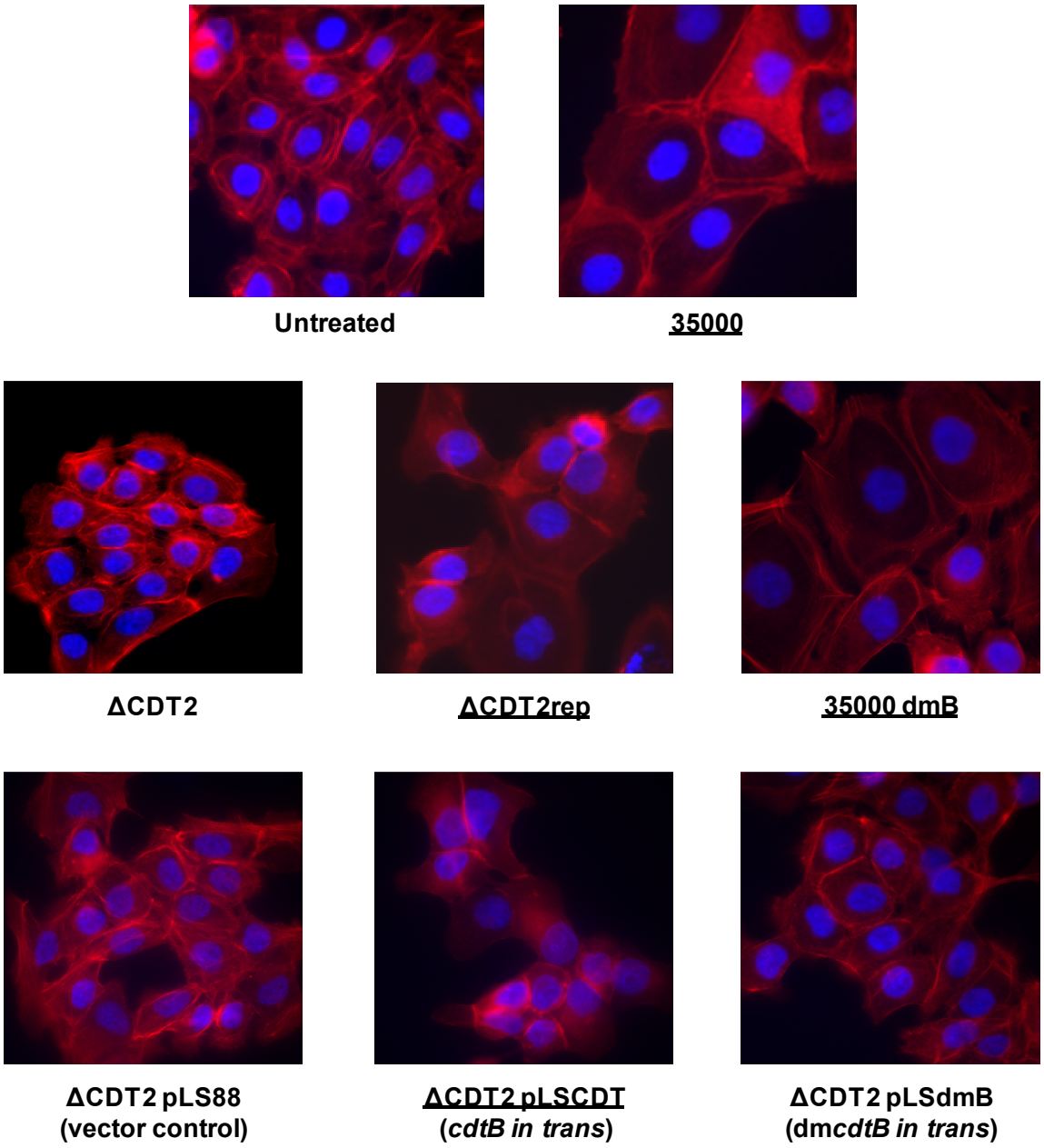

Figure 4.3. Effect of *H. ducreyi* culture supernatants on HaCaT cell morphology. Chamber slides were seeded with HaCaT cells at low confluency and treated with culture supernatant derived from the indicated *H. ducreyi* strain. After 30 hours of growth, HaCaT cells were fixed and stained with phalloidin (red) and DAPI (blue) to illuminate the overall cell structure and the cell nucleus, respectively. Underlined supernatants were applied at a 1:10 dilution; others at a 1:2 dilution.

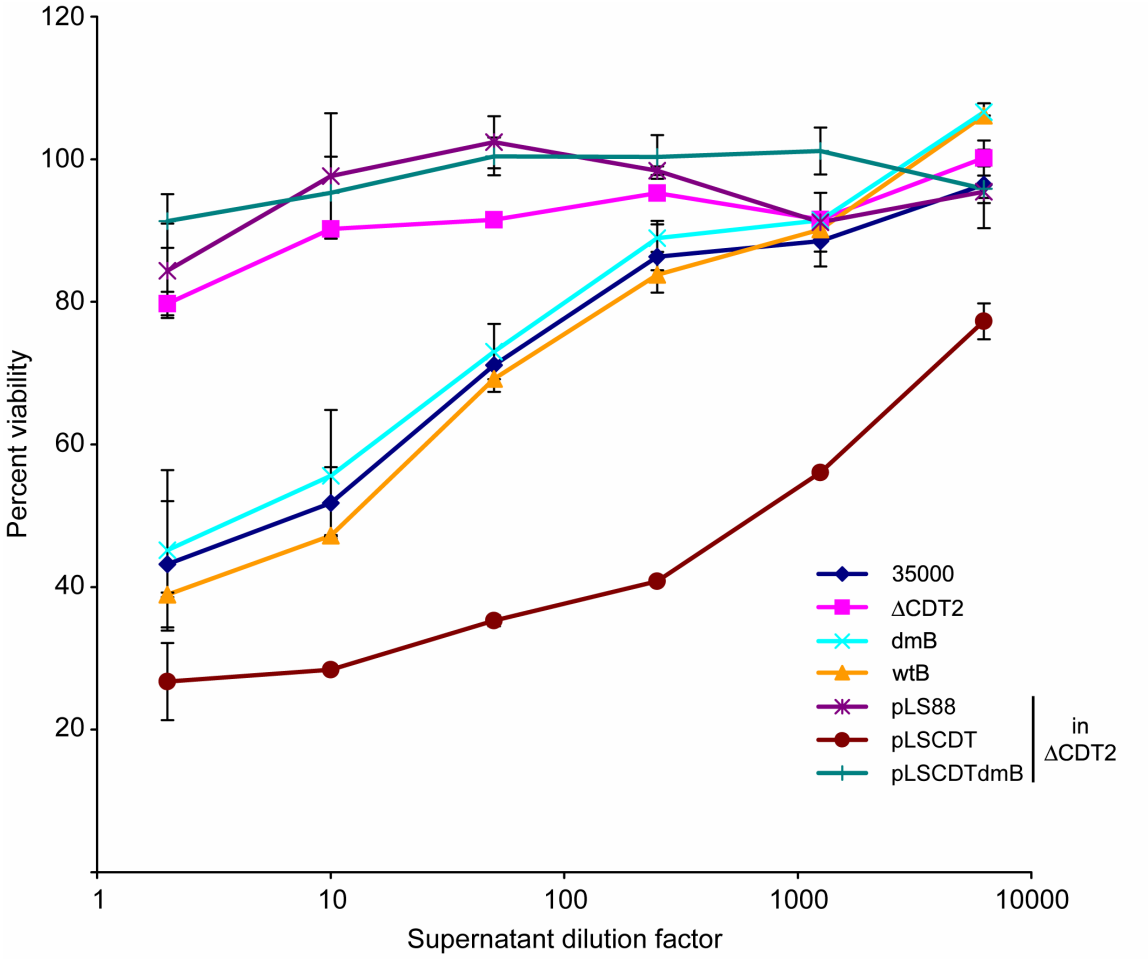

Figure 4.4. Effect of *H. ducreyi* culture supernatants on HaCat cell viability *in vitro*. HaCaT cells were exposed to culture supernatants from CDT+ and CDT- strains of *H. ducreyi* in five-fold dilutions ranging from 1:2 to 1:6250. Relative viability was measured using the fluorescent metabolic indicator dye AlamarBlue, comparing signal of supernatant-treated HaCaTs to that of untreated HaCaTs.

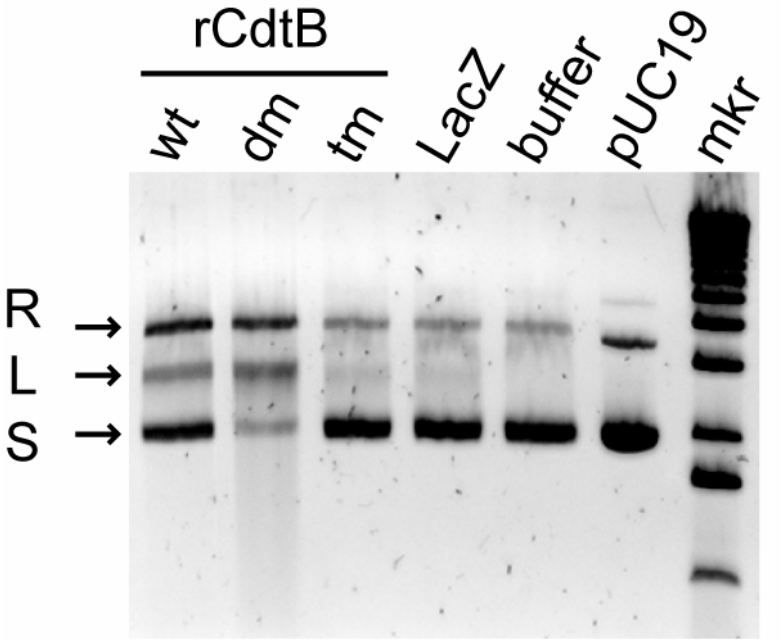

Figure 4.5. *In vitro* nuclease activity of purified recombinant *H. ducreyi* CdtB variants. Recombinant hexahistidine-tagged wild type (wt), double-mutant (dm; H160A/D199A), and triple-mutant (tm; H160A/D199A/H274A) variants of *H. ducreyi* CdtB purified from *E. coli* were incubated with supercoiled pUC19 plasmid *in vitro*. The resulting digest was resolved on agarose gel, stained with EtBr and photographed. Nicking or double-stranded cutting of the plasmid substrate are respectively illustrated by conversion of supercoiled (S) plasmid to relaxed (R) or linear (L) form. Hexahistidine-tagged LacZ expressed and purified alongside the CdtB variants served as a control to ensure that *E. coli* nucleases were excluded by the nickel affinity purification process. Buffer-only (buffer) and unincubated plasmid (pUC19) controls are also shown along with Invitrogen 1kb marker (mkr).

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CHAPTER 5

DISCUSSION

Up until the 1980s and 90s, chancroid was a highly prevalent genital ulcer disease in developing nations. In South Africa, chancroid accounted for up to 50% of GUD cases in men as recently as 1985, outnumbering cases of syphilis (*Treponema pallidum*), granuloma inguinale (*Calymmatobacterium granulomatis*), lymphogranuloma venereum (*Chlamydia trachomatis*), and herpes simplex virus (20). In many locations over the past few years, however, chancroid incidence has steadily declined. In the United States, where nearly 5000 cases of chancroid occurred in 1987, only 17 cases of chancroid were reported in 2005, which is the lowest number ever reported (7). Even in some areas of Africa where chancroid is much more prevalent than in the U. S. and Europe, clinicians have reported a distinct downward trend in the incidence of chancroid (22), with up to a 25-fold decrease between 1993 and 2003 in some cases (26). Many factors have probably contributed to this apparent recession of chancroid. More effective prevention strategies, including emphasis on genital hygiene, encouragement of condom use and simple instruction to abstain from intercourse when genital ulcers are present (on self or partner) have likely played a role in this reduction in frequency (29).

Although antibiotic-resistant isolates are commonly seen in some communities, there are still several viable and effective options for antimicrobial therapy against *Haemophilus ducreyi*. First-line therapies such as azithromycin and ceftriaxone can resolve chancroid with a single dose, eliminating low patient compliance as an obstacle to successful treatment or a source of further development of antibiotic-resistant isolates (40). Single-dose azithromycin treatment is particularly effective (36) and may render individuals refractory to reinfection with *H. ducreyi* for nearly two months (34). Access to modern health care unfortunately still presents a problem for many areas with endemic chancroid, so these often-expensive therapies are not widely available in the places most burdened with this disease (35).

Chancroid is not a lethal disease. Lesions remain localized to the skin and *H. ducreyi* has never been shown to cause systemic infection. Still, chancroid lesions are painful, potentially disfiguring, and subject their bearer to increased risk of acquiring other STI pathogens (35). Though susceptible to antibiotic therapy, *H. ducreyi* aggressively damages host skin both by manufacturing toxins as well as attracting a concentrated locus of host-generated inflammation. The effects of these bacterial toxins and components may remain within chancroid ulcers even after clearance of *H. ducreyi*, and immunocompromised individuals suffer more intense, slower-healing lesions that respond poorly to drug therapy (37, 38). Further understanding the mechanisms by which *H. ducreyi* mediates cutaneous damage may lead to the development of agents that enhance antimicrobial therapy by halting progression of ulcerative damage or speeding the resolution of ulcers in patients at risk for protracted chancroid pathology. Any strategies that reduce the length of time that chancroid

lesions remain a cofactor for STI acquisition and transmission could have a positive impact on global public health.

It is important to consider that humans are the sole vectors and victims of *Haemophilus ducreyi*; therefore, should the incidence of chancroid be suppressed to a sufficiently low state, eradication could be attainable. However, antibiotic treatment alone may not be sufficient to attain this goal, even excluding the possibility of resistance to new classes of antibiotics, for several reasons. *H. ducreyi* infection does not promote protection upon subsequent exposure, making antibiotic treatment effective for only a single instance of chancroid in a given individual. Upon re-exposure, patients require re-treatment. Areas where public health infrastructure is poor and where the commercial sex trade is widespread are fertile ground for endemic chancroid. Some researchers have even suggested that chancroid is a bellwether disease indicating communities whose social structure and policies are strongly imbalanced or ill-equipped to deal with endemic STI (29). Even in the face of the overall downward trend, the highly infectious nature of *H. ducreyi* and the episodic nature of chancroid outbreaks (for example, the U. S. experienced a surge from < 1000 to between 2000 and 5000 cases/year between 1983 and 1995) suggest that resurgence may be a likely event (7).

An effective immunization against *H. ducreyi* infection is the best strategy for eliminating chancroid. A single or few administrations of such a vaccine would render individuals refractory to acquisition or transmission of *H. ducreyi* for an extended period of time. Some common sexually-transmitted pathogens have evolved mechanisms of evading

immunity: *Neisseria gonorrhoeae* varies the antigenic character or expression state of its exposed surface proteins (21), and HIV rapidly develops resistance to antiviral compounds due to the high mutation rate of its replicative reverse transcriptase (24). These mechanisms mean that for these pathogens, any vaccine may ultimately be "outgrown" by the evolution of strains that circumvent its protective effect. Even with the advent of the whole-genome sequence of *H. ducreyi*, no evidence for systems of antigenic variation or hypermutation have been detected. Furthermore, at least six surface-exposed proteins in *H. ducreyi* are important or essential in experimental models of chancroid: DltA, DsrA, Flp, HgbA, NcaA, and Pal (2, 6, 16, 17, 19, 28). For these reasons, we submit that *H. ducreyi* may be a suitable target for vaccine design. The studies presented in this dissertation have attempted to gain insight into the means by which *H. ducreyi* causes disease, with the ultimate goal of generating effective strategies for defeating chancroid.

Evaluating the humoral immune response to *Haemophilus ducreyi*.

The studies presented in Chapter 2 investigated the extent of *H. ducreyi* evasion of the humoral immune response in the pig model of chancroid and found that repeated inoculation with a wild type *H. ducreyi* isolate does promote formation of modest but passively transferable humoral response. Although some studies of individuals with natural chancroid indicated that *H. ducreyi* serum antibodies are produced over prolonged ulcerative infection, these antibodies do not effectively fix complement to kill *H. ducreyi in vitro*. It is also improbable to locate and observe chancroid patients who have been iteratively infected

with the same strain to see if a homologous prime-and-boost produces a more effective response. The human challenge model of chancroid addressed this question by experimentally infecting volunteers twice with strain 35000, but showed that there was no significant increase in specific antibody titer after two experimental infections of 3 to 7 day duration spaced 5 to 17 months apart (3). Experimental lesions in this model cannot progress past the pustular stage because of the painful nature of ulceration. The abridged course of infection as well as the limited number of inoculations may have prevented development of a more profound immune response.

In the pig model, we inoculated animals up to four times successively and allowed lesions to develop for up to a week, at which stage overt ulcers had formed. With each round of inoculation, we saw a successive reduction in the number of bacteria recovered from each lesion with a concordant reduction in the histopathological lesion severity. This mitigation of disease symptoms was accompanied by a five-fold increase in specific serum IgG. Naïve pigs infused with immune serum then challenged with wild type *H. ducreyi* had intralesion bacterial recovery and histopathology scores similar to those of a multiply-infected pigs, and not preimmune pigs. Though the magnitude of this immune response was not large and the protection afforded by passive immunization cannot be deemed complete, this finding revealed the important fact that an antibody response is appropriate and effective against *H. ducreyi* infection. It follows that an artificially amplified immune response may produce a consonantly amplified level of protection.

Elkins *et al*. recently reported protection against experimental *H. ducreyi* challenge by vaccination in the pig model (1). Hemoglobin binding protein A (HgbA), a highly-conserved surface protein involved in iron acquisition by *H. ducreyi* (12, 13, 15, 30) and essential for its survival in the human host (2), was purified and used to immunize pigs. Compared to mock-vaccinated animals, lesion severity was dramatically reduced and recovery of *H. ducreyi* was nil. The antibody response induced by HgbA vaccination was specific, robust, bactericidal, and prevented the proper function of HgbA as an iron acquisition system, thus demonstrating the efficacy of a well-targeted, high-magnitude immune response against *H. ducreyi* challenge.

Identification of NcaA: an essential virulence factor and potential immunogen.

In Chapter 3, we detailed the characterization of NcaA, *H. ducreyi* outer membrane protein with homology to DsrA, an *H. ducreyi* OMP that provides resistance to killing by serum complement and is essential for both swine and human experimental infection (6, 8, 14). The predicted structures of NcaA and DsrA classify them as Oca proteins, which are found in several gram-negative bacteria including *Yersinia*, *Escherichia*, and *Moraxella* species, and are generally involved in adherence to extracellular matrix proteins within host tissue (27). Our results showed that, like DsrA, NcaA is one of the OMP antigens recognized by *H. ducreyi*-immune pig serum; discordantly, NcaA was not shown to be involved in serum resistance. We showed that NcaA specifically mediates binding to type I collagen, the predominant structural protein found in human skin, and *H. ducreyi* has been found

associated with collagen in both natural (5) and experimentally-induced (4) human chancroid lesions. Though we have not directly linked NcaA-mediated collagen binding to virulence, we showed that NcaA is indispensable for infection in both swine and human models of chancroid.

As a multifunctional *H. ducreyi* OMP that is essential to virulence, we submit that NcaA, much like HgbA, is a promising vaccinogen candidate. We have begun immunization studies to this effect in the pig model, but the oligomeric nature of NcaA has presented technical difficulties in expressing and purifying sufficient recombinant NcaA for immunization. Also we have been unable to assess the efficiency with which anti-NcaA antibodies are bactericidal. One potential complication with using NcaA as a vaccine subunit is that NcaA shows a modest amount of antigenic heterogeneity across "Class I" and "Class II" *H. ducreyi* species (39). This problem could be overcome by immunization with a multivalent NcaA preparation. Further, as an Oca family member, NcaA immunization could conceivably afford the benefit of cross-reactive immunity to other Oca-expressing pathogens.

Assessing the effects of CDT on *H. ducreyi* pathogenesis and host immunity.

The studies outlined in Chapters 2 and 3 addressed the presence and efficacy of an anti-*H. ducreyi* humoral response and the discovery of an *H. ducreyi* OMP that is essential to virulence and a promising vaccine target. Studies outlined in Chapter 4 were intended to explore a potential cause of the diminished immunity seen in natural *H. ducreyi* infection. *H.*

ducreyi cytolethal distending toxin (CDT) intoxicates a broad range of eukaryotic cell types present in both the skin (keratinocytes, skin fibroblasts) as well as involved in the immune response (dendritic cells, macrophages, lymphocytes) (9-11, 18, 23, 31, 32). For these reasons, respectively, we proposed that CDT may influence the ability of *H. ducreyi* to infect the skin as well as to affect adaptive immunity and the development of a protective humoral response. Two ΔCDT (*cdtABC* locus deletion) mutants created in parental strain *H. ducreyi* 35000 were profoundly attenuated in the pig model of chancroid. However, addition of the *cdtABC* locus *in cis* or *in trans* to either of these mutant strains did not restore virulence. We also designed a consecutive prime-and-boost inoculation experiment to determine whether repeated administration of CDT-deficient *H. ducreyi* enhanced immunity to wild type challenge. Pigs challenged with 35000 following either two inoculations of 35000 or two inoculations of ΔCDT1 were equally permissive to wild type infection.

Though we cannot discount the importance of CDT in *H. ducreyi* infection, lack of complementation suggests that deletion of the *cdtABC* locus selects for compensatory mutations that impart an attenuated phenotype. The fractional contributions of *cdtABC* and the other currently unidentified gene(s) to chancroid pathology, therefore, cannot be ascertained. It may be possible to locate these secondary mutations and independently measure their contribution to virulence. The effect of *H. ducreyi* CDT on the adaptive immune response is also questionable because the cryptic attenuation of the ΔCDT strains may impair their growth *in vivo* such that their numbers are insufficient to immunize their host. In light of these data, we can neither prove nor disprove our prediction that a *truly*

isogenic *cdtABC* mutant will be more immunogenic than wild type. We will continue to investigate this hypothesis in future experiments. The concluding text describes our first effort to that effect.

In an attempt to ablate CDT function without removing the entire locus (and theoretically avoiding compensatory mutation), we constructed an *H. ducreyi* mutant expressing a modified "double mutant" CdtB (dmCdtB). This mutant was intended to lack DNase activity, with the two amino acid substitutions mapping to the putative nuclease active site of CdtB (25). We found that supernatants from *H. ducreyi* DMB (35000 dm*cdtB*) are as toxigenic as wild type to cultured cells and that *in vitro* nuclease activity of recombinant dmCdtB is intact. Therefore the third attempt to create an isogenic mutant deficient in CDT activity failed. These findings were surprising because a number of studies demonstrated ablation of the same putatively catalytic amino acids in CdtB results in loss of nuclease function (25, 33). Our effort to produce *H. ducreyi* strain DMB is unique; all published studies thus far have used purified recombinant CdtB variants instead of isogenic mutants within the native organism. We also chose to make more aggressive amino acid substitutions than other researchers (our H160A, D199A as opposed to H160Q, D199S). Both of these factors may explain the persistence of nuclease activity in our strain DMB and recombinant dmCdtB preparations as opposed to other researchers' findings.

In adding a third substitution, H274A, to create the recombinant ("triple mutant") tmCdtB, we appear to have eliminated *in vitro* nuclease activity. We plan to introduce this *cdtB* allele into *H. ducreyi*. If culture supernatants derived from this strain are devoid of or

highly diminished in CDT toxicity, we will repeat our initial experiments in the swine model to ascertain the contribution of CDT to *H. ducreyi* pathogenesis.

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